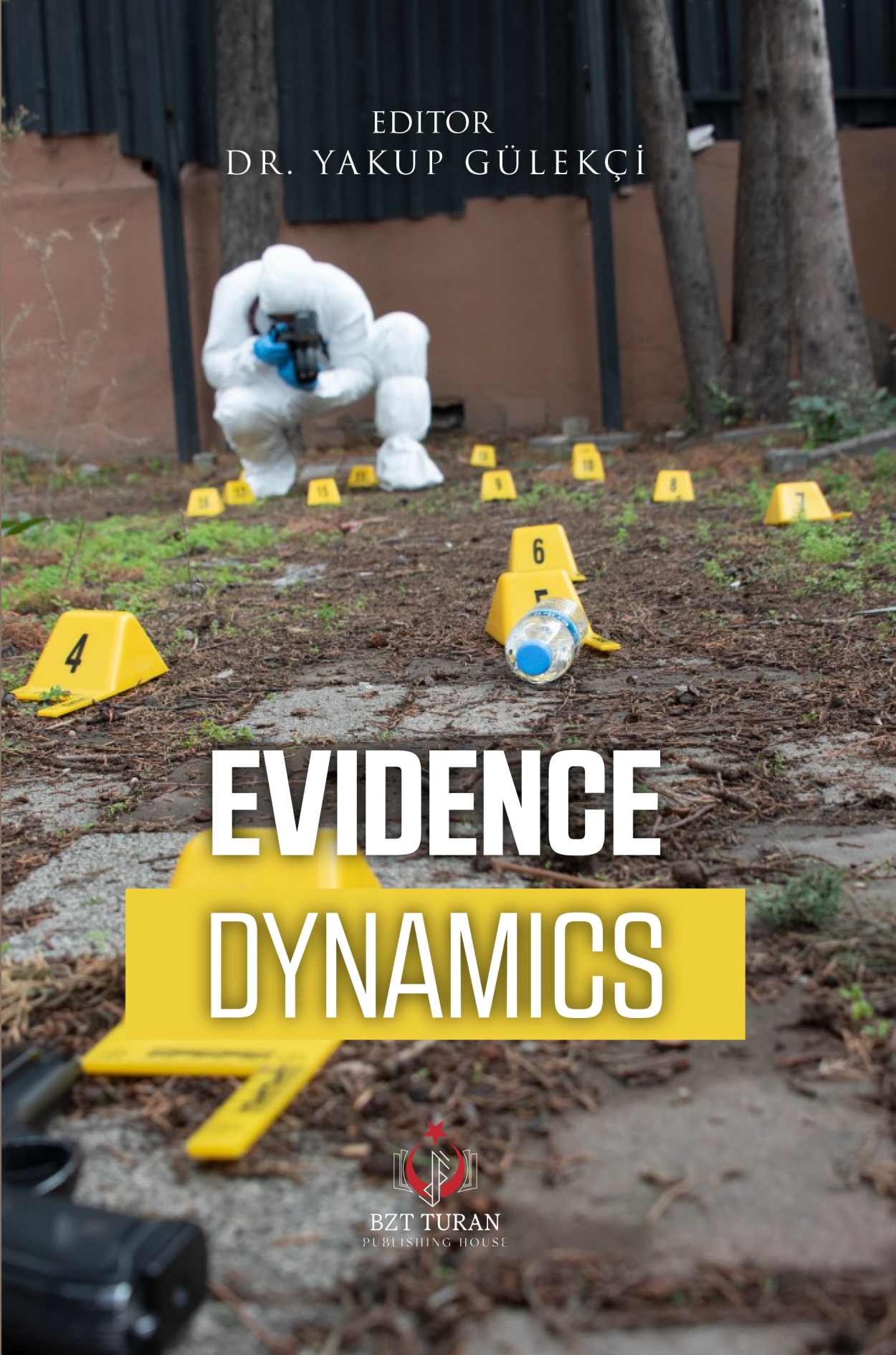
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EDITOR:
DR. YAKUP GÜLEKÇİ

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Dr. GÜLEKÇİ endeavors to transform forensic sciences into practical tools for everyday use and to develop fingerprint enhancement methods for crime scenes. He has initiated a patent study in this regard.

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Uzun yıllar olay yeri inceleme, parmak izi geliştirme laboratuvarı ve sualtı olay yeri inceleme alanlarında Emniyet Genel Müdürlüğü / Polis Kriminal Daire Başkalığı bünyesinde uzman olarak çalışan, başta "kaşıkçı" cinayeti olmak üzere pek çok dikkat çeken önemli olaylarda bilirkişilik yapan ve Kütahya Sağlık Bilimleri Üniversitesi, Adli Bilimler bölümünde öğretim üyeliğine devam eden Yakup GÜLEKÇİ, 2009-2020 yılları arasında İstanbul Emniyet Müdürlüğü'nde görev yaptı. İstanbul Olay Yeri İnceleme ekiplerinde ve İstanbul Adli Polis Laboratuvarında çalıştığı süre içinde parmak izi geliştirme yöntemleri, kan lekesi model analizi ve atışın yeniden yapılandırılması konularında geniş tecrübeye sahip oldu ve uzmanlık eğitimlerinde eğitmen olarak görev yaptı.

Lisans eğitimini 2007 yılında eğitim fakültesinde, kriminal uzmanlık eğitimlerini (olay yeri inceleme ve parmak izi arastırmaları vb.) 2012 yılında polis akademisinde tamamladı. Yüksek lisansını 2012 yılında, doktoranı ise 2017 yılında İstanbul Üniversitesi Adli Bilimler Enstitüsü'nde tamamladı. Yüksek lisans tezi "Sualtı Olgu Çalışmasından Elde Edilen Parmak İzi Delillerinin Modelleme İle Değerlendirilmesi", doktora tezi ise "Olay Yerinde Elde Edilen Patlama ve Molotof Kokteyli Atma Olayında Kullanılan El Yapımı Yangın Söndürücüler Üzerindeki Parmak İzlerinin ve Biyolojik Bulguların İncelenmesi" konularında yaptı.

Uluslararası hakemli dergilerde yayımlanan 6 adet makalesi vardır. Uluslararası bilimsel toplantılarda sunulan ve bildiri kitaplarında basılan 32 adet bildirisi vardır. 3 (üç) adet kitap editörlüğü, 6 (altı) adet kitap bölümü vardır. Ulusal hakemli dergilerde yayımlanan 10 adet makalesi vardır. İki adet dergide hakemlik yapmıştır. Bir adet sempozyum, 3 adet ulusal ve uluslararası Kongrenin bilim ve düzenleme kurullarında gören yapmıştır.

Gülekçi, çalışmalarımla adli bilimleri günlük hayatta, adli olayların çözümünde kullanılabilecek araçlara dönüştürmeye çalışmaktadır. Ayrıca; olay yerlerinde kullanılabilecek parmak izi iyileştirme yöntemleri geliştirmeye çalışıyor. Bu konuda bir patent çalışması mevcuttur.

Preface

Forensic science is an interdisciplinary field that plays a crucial role in the pursuit of truth, a fundamental requirement of justice. Rapid advancements in science and technology have introduced significant innovations in methods and techniques for solving crimes. The effective application of these innovations in forensic processes requires the continuous updating of educational and research practices. Consequently, adopting a multidisciplinary approach in forensic science while embracing innovative methods instead of relying solely on established knowledge is essential for introducing novel solutions to the evidence collection and analysis process.

Evidence Dynamics seeks to transcend the traditional understanding of forensic science by addressing the criminal phenomenon holistically, with a focus on innovative methods for evidence research. Every stage of the process from the meticulous collection and preservation of crime scene findings to their analysis through the latest technologies plays a critical role in ensuring scientific accuracy. This book thoroughly examines how new techniques and analytical methods in criminal investigations contribute to solving crimes and explores which innovative approaches can enhance the reliability of evidence.

The book does not limit itself to classical methods in evidence management but also emphasizes recent innovations, such as advanced analytical techniques and the application of artificial intelligence in forensic science. These methods not only accelerate the evidence evaluation process but also stand out as powerful tools that help ensure the proper administration of justice.

Designed primarily for students of forensic science, judges, prosecutors, and experts working in the criminal divisions of law enforcement agencies, this work provides a clear, accessible, and practical guide to evidence management. By translating theoretical knowledge into practical application, it offers a roadmap for how innovative approaches can be integrated into criminal investigations. The use of visual aids further supports the explanations, simplifying complex

concepts and illustrating how evidence can be scientifically analyzed.

In conclusion, *Evidence Dynamics* introduces an innovative perspective on evidence research in crime resolution, enabling forensic science to be applied more effectively and efficiently. We hope this book will contribute both to academic research and the practical application of forensic science, fostering the adoption and dissemination of new methodologies.

We trust that this work will serve as an inspiring resource for all researchers and professionals seeking to advance their expertise in forensic science...

Önsöz

Adli Bilimler, hukukun en temel gereksinimlerinden biri olan gerçeğin ortaya çıkarılmasına bilimsel katkı sağlayan, hızla gelişen bir disiplinler arası alandır. Bilim ve teknolojideki hızlı ilerlemeler, suçların çözümüne yönelik yöntem ve tekniklerde önemli yenilikleri beraberinde getirirken, bu yeniliklerin adli süreçlerde etkili bir şekilde uygulanması, eğitim ve araştırma süreçlerinin de sürekli güncellenmesini gerekli kılmaktadır. Bu nedenle, adli bilimler alanında multidisipliner bir yaklaşım geliştirmek, sadece geçmiş bilgilerle yetinmek yerine inovatif yöntemleri benimseyerek delillendirme sürecine yenilikçi çözümler sunmayı kaçınılmaz kılmaktadır.

Delillendirme Dinamikleri kitabı, klasik adli bilim anlayışının ötesine geçerek, suç olgusunu tüm yönleriyle ele almayı ve delil araştırmalarında inovatif yöntemleri merkeze koymayı amaçlamaktadır. Olay yerinden elde edilen bulguların titizlikle toplanması ve korunmasından başlayarak, bu bulguların en güncel teknolojilerle analiz edilmesine kadar geçen sürecin her aşaması, bilimsel doğruluğun sağlanması adına büyük önem taşır. Bu kitap, özellikle suç araştırmalarında kullanılan yeni tekniklerin ve analitik yöntemlerin suçun çözümüne nasıl katkı sağladığını detaylı bir biçimde ele almakta ve delillerin güvenilirliğini artırmak için hangi yenilikçi yaklaşımların kullanılabileceğini tartışmaktadır.

Kitap, delil yönetimi sürecinde sadece klasik yöntemlere değil, aynı zamanda son yıllarda gelişen analiz teknikleri ve yapay zekanın ileri düzeyde kullanımı gibi yenilikçi yöntemlere de geniş yer ayırmaktadır. Bu yöntemler, delillendirme sürecini hızlandırmanın yanı sıra, adaletin doğru şekilde tecelli etmesine katkı sağlayan güçlü araçlar olarak ön plana çıkmaktadır.

Özellikle adli bilimler eğitimi alan öğrencilere, hâkim ve savcılara, kolluk kuvvetlerinin kriminal birimlerinde çalışan uzmanlara yönelik hazırlanan bu eser, delillendirme süreçlerine dair açık, anlaşılır ve uygulamalı bir rehber olma niteliği taşımaktadır. Teorik bilgileri pratiğe dönüştürerek, suç araştırmalarında yenilikçi yaklaşımların nasıl uygulanacağına dair yol gösterici bir kaynak sunmayı amaçlamaktadır.

Görsellerle desteklenen anlatım, okuyuculara karmaşık kavramları sade bir dille açıklamakta ve delillerin bilimsel temellerle nasıl analiz edileceğini göstermektedir.

Sonuç olarak, *Delillendirme Dinamikleri*, suçların çözümünde delil araştırmalarına yenilikçi bir perspektif kazandırarak, adli bilimlerin daha verimli ve etkili bir şekilde uygulanmasına olanak sağlamaktadır. Bu kitabın, hem akademik çalışmalara hem de adli bilimlerin uygulama alanlarına katkıda bulunarak, yeni yöntemlerin benimsenmesine ve yaygınlaşmasına ışık tutması en büyük dileğimizdir.

Adli bilimler alanında ilerleme kaydetmek isteyen tüm araştırmacı ve uzmanlar için ilham verici bir kaynak olması temennisiyle…

CONTENTS

A NEW TECHNOLOGY IN EVIDENCE EVALUATION: THE ARTIFICIAL INTELLIGENCE REVOLUTION IN FORENSIC SCIENCES

Fatma ÇAVUŞ YONAR, Sena Nur KADEM

Chapter 1

A New Technology In Evidence Evaluation: The Artificial Intelligence Revolution In Forensic Sciences

FATMA CAVUS YONAR[1]

SENA NUR KADEM[2]

Introduction

Forensic Sciences encompass the technical services carried out to identify crimes and criminals by examining and evaluating physical evidence obtained during judicial and administrative investigations. This process also aims to establish patterns between the crime, crime scene, victim, and perpetrator and involves the application of various scientific disciplines to ensure justice (Suzanne, 2019). One of the most crucial elements of a forensic investigation is the crime scene and its related findings. When appropriately examined in solving forensic cases, these findings can lead to accurate conclusions. Crime scene investigation follows a systematic and scientific approach. The rapid developments, particularly in innovative technological approaches, have enabled more comprehensive and multi-faceted examinations of the evidence collected from crime scenes. At the forefront of these technological advancements is artificial intelligence (AI). AI refers to the branch of computer science that seeks to solve cognitive problems associated with human intelligence by perceiving specific functions in a virtual environment and subsequently performing related tasks (Luger, 2009). In this context, this section will explore current AI applications in forensic genetic analysis, forensic anthropology, forensic odontology, forensic toxicology, forensic medicine, fingerprint and crime scene investigations, forensic ballistics, and digital forensics while addressing the risks, ethical considerations, and legal aspects of AI use in forensic sciences.

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1. General Overview of Artificial Intelligence

AI has a deep-rooted history and has undergone a remarkable evolution over time. This evolutionary process has led to the emergence of various subfields within AI, forming the foundational concepts of the field. The algorithms used in AI consist of procedures that enable computer-based machines to perform human-like tasks, learn from experiences, and adapt to new inputs, helping computers solve problems on various scales (Bengio & LeCun, 2007). Each subfield and its algorithms contribute to the overall functioning of AI by offering unique techniques and methods. Among these subfields, machine learning, deep learning, and natural language processing stand out as key areas.

1.1. Machine learning (ML)

ML (Machine Learning) is a comprehensive field focused on creating predictive models or identifying groups within data. At its core, ML aims to objectively replicate the human ability to recognize specific patterns through computational calculations. This field becomes particularly relevant when the dataset being analyzed is either very large or highly complex, automating the data analysis process and making it repeatable (Greener et al., 2021). ML algorithms play a crucial role in all these processes and are divided into three main categories: supervised, unsupervised, and reinforcement learning (Bishop, 2006; Sarker, 2021). Supervised learning is based on an existing input-output set and is used to generate appropriate outputs for previously unseen data. In unsupervised learning, only input data is available, and algorithms are trained to understand patterns within this data, which can also be used for clustering and detecting anomalies in datasets. Reinforcement learning enables software to learn through interactions with its environment and is based on the trial-and-error principle, where responses to encountered situations lead to rewards or penalties (Han et al., 2022; Mohammed et al., 2016; Stone, 2023). ML models support each of these processes, and the most widely used ML models include:

- ✓ Logistic Regression (LR): LR is a statistical model that uses the logistic (sigmoid) function to estimate probabilities (Cessie & Houwelingen, 1992; Pedregosa et al., 2011; Peng et

al., 2002). This model predicts dependent variables and models the relationship between them using independent variables as input parameters. LR is commonly employed to solve classification problems but can also be effectively used for regression tasks.

✓ Naive Bayes (NB): NB is based on Bayes' theorem and calculates probabilities from data. It is a classification algorithm that is easy to understand, fast to train, and widely used. NB provides effective results in both binary and multi-class classification tasks such as document or text classification and spam filtering. It is particularly useful for classifying noisy data and developing reliable predictive models (Sarker, 2019; Zhang, 2004).

✓ k-Nearest Neighbors (KNN): KNN is an algorithm based on distance calculations and is used for classification or regression tasks (Harrington, 2012). KNN predicts the class of a target variable by considering which class is most common among its nearest neighbors, based on a vector of independent variables (Kramer, 2013). Although simple and easy to interpret, KNN requires large datasets for optimal performance.

✓ Decision Trees (DT) and Random Forest (RF): DT aims to build a model capable of predicting the value of a target variable by learning decision rules from features. It is used for both classification and regression tasks (Pedregosa et al., 2011). Notable DT algorithms include ID3 (Quinlan, 1986) and C4.5 (Quinlan, 1993). On the other hand, RF creates multiple decision tree classifiers in parallel over different data subsets and uses majority voting or average values for the final result. This approach reduces the risk of overfitting while improving prediction accuracy (Hastie et al., 2009; Liu et al., 2012; Pedregosa et al., 2011).

✓ Support Vector Machines (SVM): SVM is used for regression or classification tasks and aims to find the optimal classification function that best distinguishes between members of two classes (Suthaharan, 2016).

✓ Artificial Neural Networks (ANN): ANN is an ML algorithm inspired by biological neural networks designed to mimic the brain’s learning and pattern recognition functions. It consists of interconnected artificial neurons (nodes) that transmit signals to one another. These nodes process incoming signals using mathematical operations

such as weighted sums and activation functions, representing a simplified model of how neurons in the brain communicate (Stone, 2023; Zou et al., 2008). ANN typically includes an input layer, an output layer, and a series of hidden layers between them (Hastie et al., 2009). The input layer receives raw data, which is processed as it moves through each layer of the network, with the output layer generating a prediction or decision. Since the internal layers are often unobservable, they are referred to as hidden layers. Additionally, various types of ANNs exist, such as multi-layer perceptrons (MLP) and convolutional neural networks (CNN). In conclusion, ANN stands out as a powerful ML tool capable of delivering high performance in many tasks due to its ability to extract features from complex datasets (Stone, 2023).

1.2. Deep learning (DL)

DL, a subset of ML models based on artificial neural networks (ANN), focuses on representation learning (Han et al., 2022). The DL model consists of a neural network with many layers and parameters, with most DL techniques utilizing neural network architectures. As a result, these networks are often referred to as "deep neural networks (DNN)" (Shinde & Shah, 2018). In DL models, layers closer to the data input learn basic features, while the higher layers learn more complex features derived from these simpler ones. Thus, DL offers a practical approach for extracting and analyzing meaningful information from large datasets obtained from various sources (Zhang et al., 2018). DL presents several advantages over traditional ML. For instance, the outcomes achieved through DL are far superior to those produced by conventional ML methods because DL, unlike ML, does not require manually extracted features; instead, it automatically extracts, processes, and makes decisions based on raw data (Aloysius & Geetha, 2017; Lecun et al., 2015; Setiowati et al., 2017). DL algorithms further enhance DL's feature extraction and processing ability. While there are several DL algorithms, the most commonly used ones are:

- ✓ Convolutional Neural Network (CNN or ConvNet): CNN is inspired by the animal visual cortex and is one of the most well-known algorithms in DL (Krizhevsky et al., 2017; Lecun et al., 2015; Li et al., 2021; Zhou, 2020). CNN is composed of convolutional layers, pooling layers, and fully connected

layers (Chauhan & Singh, 2019). CNNs effectively utilize the two-dimensional structure of input data and are widely used in tasks such as image and video analysis (Amerini et al., 2019; Dal Cortivo et al., 2021), image processing and categorization (Hossain & Sajib, 2019; X. Liu et al., 2023; Parab & Mehendale, 2021; Tayal et al., 2022), and text and facial recognition (Chahi et al., 2023; Lokeshnaik et al., 2023; Said et al., 2020; Vankadaru et al., 2023; Venkateswarlu & Ch, 2024; Xing & Qiao, 2016). With its advantageous features, CNN delivers higher accuracy and enhances system performance (Dargan et al., 2020; Ongsulee, 2018). Moreover, CNN's ability to automatically detect important features is considered one of the aspects that make it more powerful than traditional ANN (Sarker, 2021).

✓ Recurrent Neural Networks (RNN): RNN is a type of DNN known for memorizing and retaining previous data, which it uses to inform the current output. It is called recurrent because it performs the same operations for each sequence member, with the results depending on previous computations (Kaur & Mohta, 2019; Tsantekidis et al., 2022). Advanced versions of RNN, such as Long-Short Term Memory (LSTM) and Gated Recurrent Unit (GRU), were introduced to overcome issues like vanishing and exploding gradients in RNNs (Tsantekidis et al., 2022). RNN is used to address sequential or temporal problems such as speech recognition (Nassif et al., 2019), image recognition (Dhruv & Naskar, 2020), natural language processing (Fathi & Maleki Shoja, 2018; Pattanayak, 2017), and text generation (Milanova et al., 2019; Souri et al., 2018).

✓ Multilayer Perceptron (MLP): MLP is a feedforward neural network consisting of an input layer, one or more hidden layers, and an output layer. This structure is used to distinguish non-linearly separable data by utilizing the backpropagation technique (Pedregosa et al., 2011; Sarker, 2021). MLPs are widely used in various fields, such as image recognition (Biswas & Mia, 2015), speech recognition (Javaheri, 2021), and natural language processing (Goyal et al., 2018; Wang et al., 2022). The flexibility of its architecture and the ability to approximate any function under certain conditions make these neural networks a central element in DL and neural network research (Deng, 2014).

1.3. Natural language processing (NLP)

NLP (Natural Language Processing) is defined as a subfield of AI that enables computers to understand texts or words written in human languages. NLP is divided into two categories: Natural Language Understanding (NLU-Linguistics) and Natural Language Generation (NLG). NLU allows machines to analyze natural language by breaking it down and examining elements such as concepts, entities, sentiments, and keywords. This field encompasses various disciplines, including phonology (which studies sound), morphology (which deals with word structures), syntax (which analyzes sentence structure), and pragmatics (which investigates contexts related to the meaning and use of language). On the other hand, NLG, as part of NLP, is the process of generating meaningful texts, sentences, and paragraphs from internal representations (Chowdhary, 2020; Khurana et al., 2023). In this context, NLP involves both understanding human language and producing meaningful content (Stone, 2023). Additionally, it is widely used in applications such as sentiment analysis (Hasan et al., 2019; Rajput, 2020), machine translation (Jiang & Lu, 2020; Khan et al., 2020), and speech recognition (Chadha et al., 2015; Rani et al., 2017).

2. The Use of AI in Forensic Sciences

Innovative technological advancements have integrated AI into the methodologies applied in forensic sciences (Figure 1). These technologies are now being employed in forensic sciences to overcome the complexities of challenging criminal investigations, accelerate the analysis of evidence, clarify cases more efficiently, and achieve more accurate results.

Figure 1. Applications of AI in forensic sciences

2.1. AI Applications in Crime Scene Investigation

Crime scene investigation plays a critical role in determining the course of a criminal investigation. Proper collection, preservation, and analysis of evidence during crime scene investigations directly impact solving crimes and ensuring justice. However, traditional methods used in crime scene investigations largely rely on the experience of experts and the characteristics of the evidence collected. This process is generally complex and time-consuming (Kaur et al., 2022; Knes et al., 2024). At this point, AI technologies have been introduced to enhance the efficiency of crime scene investigations and minimize human error (Sacco et al., 2024).

Crime scene investigations often focus on biological evidence, with blood or bloodstains being the most crucial types of evidence (Fonseca et al., 2022). One of the key questions in analyzing blood/bloodstain samples collected from a crime scene is determining the species origin of the blood (Doty & Lednev, 2018; Mistek-Morabito & Lednev, 2020). Traditional methods to distinguish human blood cells from other species are labor-intensive and require qualified experts. This creates an opportunity for AI implementation (Kislov et al., 2023). Using deep machine-learning techniques, researchers have explored the differentiation of human blood cells from those of cattle, goats, and chickens through microscopic image analysis (Shah et al., 2024). 1,955

Giemsa-stained images were analyzed using well-known algorithms such as VGG16, ResNet18, and ResNet34. The results demonstrated exceptional performance, with F1 scores and Matthews correlation coefficients indicating excellent classification for most species. This underscores AI's potential to accurately determine the origin of blood as biological evidence in forensic investigations.

The increasing volume, diversity, and complexity of data have made traditional manual cataloging, labeling, and content searching processes in crime scene image databases more challenging for forensic experts (Abraham et al., 2021; Bollé et al., 2020; Quick & Choo, 2014). In this context, Abraham et al. (2021) examined the application of two ML models, "Bag of Visual Words (BoVW) combined with SVM" and "Hierarchical CNN (Tree-CNN)," for the automatic classification of crime scene images in a dataset of 97,287 drug-related images from the Australian Federal Police (Abraham et al., 2021). The results showed that the Tree-CNN model significantly outperformed the SVM model, achieving a higher average true positive classification rate. These findings suggest that automated classification systems can potentially improve the management of large-scale image databases and offer substantial promise for real-world forensic applications.

Object detection at crime scenes is another important area of research. Object detection aims to identify and categorize objects in still or moving images (Bathija & Sharma, 2019; T. J. Nandhini & Thinakaran, 2023). Semantic object detection uses various geometric patterns to recognize objects (Ren et al., 2017; Zhu et al., 2020). However, the diversity of object shapes and viewpoints can make this process challenging. On the other hand, DL methods attempt to overcome these challenges by improving object recognition accuracy (Selvaraj et al., 2019). Researchers aiming to enhance object detection at crime scenes using advanced DL techniques and infrared (IR) imaging have employed various CNN architectures, such as Faster R-CNN and U2-Net, to improve the detection accuracy of objects like blood and weapons (Nandhini & Thinakaran, 2023). Experimental results showed that the developed CNN architecture significantly outperformed existing

models in terms of accuracy, achieving high precision and recall rates for different object classes. The study also highlighted that GPU usage could reduce computation time by up to 90%, improving operational efficiency. The findings revealed that the model effectively categorized crime scene images and provided a valuable tool for security and surveillance applications. The successes in object detection have also extended to more complex applications, such as crime scene detection.

Detecting criminal activities through camera footage is crucial; however, current systems do not facilitate a rapid response. After a crime occurs, law enforcement agencies collect and analyze video recordings, which slows down the process (Zhang et al., 2018). To address this issue, Nandhini and Thinakaran (2023) focused on using DNN for automatic crime scene detection by developing a system that identifies weapons and blood as biological evidence in images to determine whether a crime has occurred (Nandhini & Thinakaran, 2023). Various neural network techniques, such as CNN and TensorFlow, were used in the study, and the developed system achieved an accuracy rate of approximately 92.1%. The results demonstrated success in detecting knives and blood but showed difficulties in accurately classifying different types of firearms. As the training data increased, the accuracy rates for detecting blood and knives significantly improved, reaching 98.5%. The researchers concluded that their DNN-based approach could significantly enhance crime scene detection and provide law enforcement with a valuable tool for responding to potential threats quickly and effectively.

2.2. AI Applications in Forensic Genetic Analysis

Forensic genetic analysis aims to identify evidence using DNA data obtained from biological samples in criminal cases (Kowalczyk et al., 2018; Morling, 2004). However, traditional methods in the field face certain limitations, especially when obtaining DNA from degraded biological samples. As a result, AI technologies have recently been introduced in forensic genetic analyses (Galante et al., 2023; Sessa et al., 2024).

In forensic science, DNA profiling plays a critical role in criminal

investigations, paternity testing, identifying disaster victims, and locating missing persons (Alamoudi et al., 2020; Butler, 2010). The primary task in DNA profiling is to determine the number of contributors to a mixed DNA profile. However, issues like allele dropout, stutter, or artifact peaks in DNA profiles complicate the determination of contributor numbers (Alamoudi et al., 2020; Benschop et al., 2019; Taylor et al., 2014; Veldhuis et al., 2022). In this context, a software tool called TAWSEEM was developed, using an MLP DL model to accurately estimate the number of contributors to DNA mixture profiles by leveraging the PROVEDIt dataset, which is the largest publicly available DNA profile collection (Alotaibi et al., 2022). The developed DL model was evaluated using four performance metrics—accuracy, F1 score, recall, and precision—and achieved an accuracy rate of 97% on the PROVEDIt dataset.

In forensic laboratories, DNA analyses are typically based on STR profiling performed through capillary electrophoresis. However, this method has limitations when distinguishing between identical twins and analyzing small amounts of DNA samples (Carratto et al., 2022). Therefore, technologies like next-generation sequencing (NGS) are increasingly being used to obtain more comprehensive data from DNA in forensic cases. NGS allows researchers to sequence large amounts of genetic material quickly and cost-effectively, but it generates large volumes of data that complicate precise analysis (Behjati & Tarpey, 2013; Ouanes, 2024). AI and its subfield, ML, have been actively utilized to address this issue by processing NGS data more efficiently. Additionally, AI algorithms have been employed to filter out artifact peaks, significantly improving the reliability and quality of the results (Ouanes, 2024; Schmidt & Hildebrandt, 2021; Tarozzi, 2024).

In forensic cases, determining a person's biological age is particularly important for identification purposes (Schmeling et al., 2016). In this process, analyzing biomarkers like DNA methylation rates has enabled significant advancements in age estimation using AI. For instance, in a study by Vidaki et al. (2017), 45 CpG regions associated with age were selected and analyzed using Illumina's MiSeq platform from 1,156 whole blood

samples (Vidaki et al., 2017). It was found that 23 of the selected CpG regions significantly contributed to age estimation modeling, and multiple regression analysis based on these biomarkers allowed accurate prediction of biological age with an error margin of ±4.6 years. Furthermore, when a generalized regression neural network model, a type of ML, was applied, the accuracy of age prediction improved significantly, reducing the error margin to ±3.3 years.

Today, haplogroup naming is performed hierarchically using Y-SNP analysis (Hammer, 2002). However, the limited polymorphism of Y-SNPs makes it challenging and costly to simultaneously determine haplogroups and paternal lineages. Conversely, Y-STRs have the ability to identify paternal lineages and distinguish individuals within the same lineage, making them highly valuable in forensic applications (Liu et al., 2021; Zhou et al., 2020, 2022). Researchers have, therefore, begun investigating the possibility of using Y-STRs to predict haplogroups, and various software tools have been developed in this area. However, existing software tools are not capable of haplogroup prediction for genes with multi-allelic and fractional alleles. To address these limitations, a software tool called Y-Haplogroup Predictor (YHP) was developed, which uses Y chromosome short tandem repeats (Y-STRs) to predict male lineages with high accuracy (Song et al., 2024). Six ML approaches—KNN, NB, LR, SVM, DT, and RF—were used for high-accuracy haplogroup prediction. Using a dataset of 4,064 samples across 219 haplogroups, YHP achieved an impressive accuracy rate of 92.3% with the RF algorithm. This algorithm has enabled more precise lineage tracking, which is critical in forensic investigations.

2.3. AI Applications in Forensic Anthropology

Forensic anthropology is a field where human skeletal remains, severely fragmented bodies, or body parts are analyzed using scientific techniques, particularly for identification purposes in cases such as natural disasters, wars, or mass graves (Klepinger, 2006; Sledzik & Mundorff, 2016). Forensic anthropological analyses contribute significantly to investigations and the clarification of forensic cases by determining characteristics such as

ethnicity, age, height, and sex (Çeker et al., 2022; Nikita & Nikitas, 2020).

One key application of AI in the identification process involves sex, ancestry, and age estimation. A study was conducted to predict the sex of 2,141 individuals based on four different long bones—humerus, radius, femur, and tibia (Knecht et al., 2023). Five ML models were used: Linear Discriminant Analysis (LDA), Penalized Logistic Regression (PLR), RF, SVM, and ANN. The RF method yielded the best results with an accuracy rate of 92%, highlighting the effectiveness and accuracy of ML methods in predicting sex from long bones. Nikita and Nikitas (2020) also examined the efficacy of various statistical and ML classification methods for sex and ancestry prediction based on skeletal remains in forensic anthropology (Nikita & Nikitas, 2020). In their study, traditional methods like Binary Logistic Regression (BLR) and LDA were compared to ML techniques such as NB, DT, SVM, ANN, RF, Multivariate Adaptive Regression Splines (MARS), and Extreme Gradient Boosting (XGB). The results showed that LDA and linear SVM generally provided the highest accuracy, while methods like NBC and DT performed poorly.

AI also plays a crucial role in forensic facial reconstruction. In a study evaluating the accuracy of facial reconstructions based on craniofacial anthropometric measurements of the adult Egyptian population, AI-assisted processing of data obtained from three-dimensional facial templates and computed tomography (CT) scans aimed to produce more accurate and reliable reconstructions (Abdou et al., 2024). In this study, facial templates were placed on 30 skulls scanned via CT, and three-dimensional faces were reconstructed. The accuracy of these reconstructions was evaluated based on subjective facial similarity scores and objective surface distance differences. The results showed no significant differences in rankings across different subjective and objective tests for each case.

CT scans are a significant component of AI applications in forensic anthropology. A study examining how CT scans are prepared for ML applications in forensic and virtual anthropology emphasized the importance of preprocessing, normalization, and segmentation

techniques in enhancing CT scan quality and improving ML algorithms' accuracy (Lo et al., 2023). Additionally, the study highlighted the impact of these techniques on the performance of DL and other ML models and their advantages for forensic anthropology applications. Comparative radiography, a method used in forensic identification, involves comparing skeletal structures in ante-mortem and post-mortem images, which requires time-consuming and detailed analyses. Researchers have proposed innovative approaches to automate comparative radiography (CR) in forensic identification (Gómez et al., 2024). The proposed approach aimed to improve the efficiency of the candidate shortlisting process by using DL for image segmentation and evolutionary algorithms for image registration. The results showed that the approach automatically eliminated 40% of candidates through segmentation and reduced potential matches by 73% with manual segmentation. The study emphasized the potential of AI to assist forensic anthropologists in making informed identification decisions while reducing the time and effort required for manual comparisons.

2.4. AI Applications in Forensic Odontology

Forensic odontology is a field focused on identifying individuals using remains that often include teeth and jawbones (Johnson et al., 2018; Khanagar et al., 2021). Playing a crucial role in the identification of human remains during mass disasters such as fires, earthquakes, and terrorist attacks, forensic odontology has made significant advancements with the development of AI (Patil et al., 2020). AI technology in forensic odontology is used for various purposes, such as determining age and sex based on dental characteristics, identifying individuals, and characterizing bite marks (Galante et al., 2023).

Forensic odontologists are experts in identifying victims by manually comparing ante-mortem and post-mortem dental records to find matches. However, this manual comparison process can be time-consuming and increase the risk of error. To address this issue, Sathya and Neelaveni (2020) aimed to automate human identification based on dental characteristics and make the process

more efficient using DL techniques (Sathya & Neelaveni, 2020). They proposed a three-stage transfer learning approach based on the AlexNet architecture, which involved determining the position of the tooth in the jaw, classifying the tooth type, and numbering the tooth according to the universal numbering system. The study analyzed 3,159 teeth from panoramic radiographs of 106 individuals and found that the proposed method performed more accurately than classical approaches.

A study testing the effectiveness of ML methods in predicting sex using a combination of mandibular and dental dimensions analyzed lateral cephalograms and dental casts from 108 individuals (Küchler et al., 2024). Researchers evaluated the predictive capabilities of specific mandibular and dental dimensions using various algorithms, including LR, XGB classifier, KNN, SVM, MLP, DT and RF classifier. The findings revealed that mandibular ramus height and the mesiodistal dimension of the lower first molar were particularly effective in predicting sex, with the LR model providing the highest accuracy (AUC: 0.84). Despite some limitations, the study suggested that integrating mandibular and dental measurements could improve the accuracy of sex determination.

In forensic odontological age estimation, traditional methods become less effective after age 24, leading to difficulties in estimating the age of older individuals. To overcome these limitations, studies have emphasized the need for innovative solutions such as ML (Fernandes et al., 2011; Oliveira et al., 2024). One study explored an effective and non-invasive approach to human age estimation by integrating panoramic radiographs with ML techniques. It used panoramic radiographs of 12,827 teeth representing the Brazilian population, with ages ranging from 2.25 to 96.50 years, and applied a model adapted from the InceptionV4 framework for image analysis (Oliveira et al., 2024). The model produced reliable results, achieving a Mean Absolute Error (MAE) test of 3.1 ± 0.18 years and an R-squared value of 95.5%. Moreover, the model successfully used anatomical information from the mandible, maxillary sinus, and vertebrae to perform well even in edentulous cases. This study highlighted the potential of AI in advancing forensic age estimation

methods, offering a universal and efficient solution.

Another important application of AI in forensic odontology is the identification of bite marks. Bite marks play a critical role in obtaining biometric data from individuals at a crime scene, and their accurate analysis can significantly aid in solving crimes. In this context, Chandramouleeswaran and Puviarasan (2022) presented an innovative approach for bite mark analysis and classification using a deep CNN based on the Xception model (Chandramouleeswaran & Puviarasan, 2022). The study involved preprocessing images, segmenting them using the Chan Vese approach, extracting features with the Xception model, and classifying them using ML techniques like SVM and LR. The findings demonstrated that the Xception-LR model performed exceptionally well, with an accuracy rate of 96.75%, while the Xception-SVM model achieved an accuracy rate of 90.91%. These results showed that the proposed model outperformed existing methods and provided high accuracy rates in bite mark classification.

2.5. AI Applications in Forensic Toxicology

Toxicology is a scientific study that aims to identify toxic substances, investigate the process of poisoning, and predict and prevent the effects and damage caused by exposure to toxins. Forensic toxicology, a multidisciplinary field, leverages toxicology to investigate substances with toxic effects and analyze intentional or accidental poisoning cases, including the substances involved and their outcomes (Negrusz & Cooper, 2013). In recent years, research in forensic toxicology has experienced significant transformation, gaining new dimensions through the innovative possibilities offered by AI. AI applications in forensic toxicology encompass a wide range of areas, from identifying and analyzing toxic substances to diagnosing poisoning cases (Piraianu et al., 2023).

The growing popularity of new psychoactive substances (NPS) poses significant challenges in drug detection and regulation due to their molecular structures being designed to evade traditional detection methods. Gas chromatography-mass spectrometry (GC-MS) is a commonly used technique

for identifying these substances; however, the limited scope of current databases restricts its effectiveness in covering the broad spectrum of potential NPS. To address this limitation, ML models, including ANN, CNN, and balanced random forests (BRF), have been developed (Wong et al., 2023). These models were trained on 891 GC-MS spectra. The BRF model demonstrated effectiveness, achieving a macro-F1 score of 0.9 across various NPS classes, such as cathinones and cannabinoids, highlighting its potential as a powerful tool for identifying unknown NPS. Researchers emphasized that ML methods could serve as crucial tools in identifying and regulating NPS. In another study, an innovative AI-assisted system called PS2MS was designed to identify NPS using mass spectrometry (Lin et al., 2024). PS2MS creates an artificial database of potential NPS derivatives based on known substances and uses DL to generate chemical fingerprints and mass spectra. The system matches unknown substances' spectra with the artificial database to identify them. The results showed that PS2MS successfully identified cathinone derivatives in real samples, enhancing the effectiveness of illegal substance identification and presenting a valuable tool for forensic experts.

The increasing use of cannabis, along with its easy access, varying legal regulations, and the differentiation of cannabinoid molecular structures in narcotic substances produced in illicit laboratories, has intensified the importance of analytical techniques. Grijalva et al. (2024) investigated Raman microscopy, density functional theory (DFT), chemometric methods, and an innovative AI approach for analyzing basic cannabinoids (Grijalva et al., 2024). The study focused on collecting high-quality Raman spectra of seven cannabinoid standards, including delta-9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), and using classification methods such as LDA and CNN for differentiation. The results revealed a high classification success, with the LDA method achieving a 99.83% accuracy rate and the AI model providing 100% accuracy in identifying cannabinoids. Integrating advanced spectroscopic techniques with ML proved effective in reliably distinguishing cannabinoids.

In accident cases, determining whether a driver's blood alcohol

concentration exceeded legal limits at the time of death and whether alcohol was consumed shortly before driving is critical. Additionally, assessing whether the alcohol detected in toxicological samples resulted from consumption or post-mortem formation is another key aspect. To improve the interpretation of blood alcohol concentrations (BAC) in drivers involved in accidents, a Bayesian network model was developed (Maskell & Jackson, 2020). The model considered BAC along with metabolites such as 5-HTOL and 5-HIAA. The findings indicated that the Bayesian network could be an effective tool for assessing the likelihood of alcohol consumption and its impact on the driver's performance at the time of the crash.

2.6. AI Applications in Forensic Medicine

Forensic medicine involves using medical knowledge, skills, and expertise for judicial and legal purposes, including criminal cases (Vodanović et al., 2023). Forensic medicine provides law enforcement with analytical reports on issues such as determining the cause of death and estimating the post-mortem interval (PMI). However, traditional methods used in forensic medicine require expert evaluation, and the process is often complex and time-consuming. To overcome these limitations, AI technologies have begun to be applied in forensic medicine (Pathirannahalage et al., 2022).

Sudden cardiac death (SCD) is defined as an unexpected natural death caused by cardiac reasons, occurring within 1 hour of the onset of acute symptoms or within 24 hours in cases without witnesses (Khairy et al., 2022). In sudden death cases, differentiating SCD from other causes of death can be challenging when an autopsy is not performed. Additionally, traditional methods for detecting SCD involve time-consuming, costly, and complex procedures. In this context, Zhang et al. (2024) analyzed blood samples from 77 cases of SCD and non-SCD using ATR-FTIR spectroscopy and applied various ML algorithms, including SVM, RF, KNN, and ANN, to develop a diagnostic model (Zhang et al., 2024). When combined with additional cardiac indicators, the SVM algorithm achieved 100% accuracy, demonstrating the highest success rate. This innovative approach was found to offer a promising method for rapid and non-destructive diagnosis.

Characterizing gunshot wounds (GSWs) is a critical responsibility for forensic pathologists, as it can have legal consequences (Denton et al., 2006). In identifying entrance and exit wounds caused by firearms, characteristics such as muzzle imprint marks, surrounding tissue tears, bone deflection, and irregular wound edges are considered (Große Perdekamp et al., 2005). However, some cases can be more complex, making it more challenging for experts to identify the wounds. A study by Cheng et al. (2024) investigated the applicability of DL models, particularly CNNs, in classifying gunshot wounds in digital images (Cheng et al., 2024). Using a dataset of 2,418 images, the ConvNext Tiny model was trained and achieved an accuracy rate of 92.6%, with a test set accuracy of 87.99%. The model successfully demonstrated its ability to distinguish between entrance and exit wounds, with performance indicators similar to those of experienced forensic pathologists. However, the classification performance of AI decreased in atypical cases or those with multiple wounds, highlighting the importance of combining AI capabilities with human expertise.

Diagnosing drowning is one of the most challenging assessments for forensic pathologists (Ogawara et al., 2023; Spitz et al., 2006). Post-mortem CT scans have revealed imaging characteristics of the lungs in drowning cases. However, distinguishing subtle differences in CT images can be particularly difficult for less experienced forensic pathologists. A study by Ogawara et al. (2023) examined the use of AI in post-mortem CT images for diagnosing drowning cases (Ogawara et al., 2023). Researchers utilized a modified AlexNet DL model to analyze high-resolution CT scans from 153 drowning cases and 160 non-drowning cases. AI exhibited impressive diagnostic performance with an AUC of 0.95, demonstrating its potential as a valuable tool for forensic pathologists. Despite the positive results, caution was advised in interpreting cases involving CPR or the "honeycomb lung" morphology. The study highlighted the potential of AI as a promising tool in forensic medicine and emphasized the need for further research to improve AI’s application in diagnosing drowning.

Post-mortem microbial communities can be used to estimate the PMI, determine the location and cause of death, and identify human remains (Franzosa et al., 2015; Lee et al., 2017; Wu et al., 2024; Zhang et al., 2021). The human gut microbiota is generally stable, resistant to environmental factors, and rapidly proliferates after death (Can et al., 2014; Hu et al., 2021). In this context, Hu et al. (2021) investigated the usability of human gut microbiome data from the appendix and transverse colon for PMI estimation using the RF algorithm (Hu et al., 2021). Researchers analyzed gut microbiota samples from cadavers with PMIs ranging from 5 to 192 hours and found that the appendix showed predictable, sequential changes during decomposition, making it a promising sampling site. The RF regression model revealed a strong correlation between the appendix microbiome and PMI, with a mean absolute error of approximately 25.79 ± 0.43 hours. However, the study also noted limitations, such as the short observation period and the influence of environmental factors on microbial communities. The study demonstrated that the appendix microbiome could serve as a valuable tool for PMI estimation in forensic science and emphasized the need for more advanced ML techniques to improve the accuracy of PMI predictions in forensic investigations.

2.7. AI Applications in Fingerprint Analysis

Fingerprints are considered one of the oldest and most established biometric identification methods due to their unique, immutable, and classifiable characteristics (Saferstein, 2014). Since every individual has distinctive fingerprints, they play a crucial role in personal identification and criminal investigations (Win et al., 2020). Advances in AI technology have also impacted fingerprint analysis and recognition processes, with various ML methods now being used for detecting, visualizing, classifying, and analyzing fingerprints.

When a finger touches a hard surface, secretions from the skin leave characteristic ridge patterns on the surface. These patterns are usually invisible to the naked eye and are therefore referred to as latent fingerprints (LFP) (Hazarika & Russell, 2012). To make LFPs visible, chemical

or physical processes that interact with the skin secretions and enhance the contrast of the prints are required (Balsan et al., 2019). To improve the detection and identification of LFPs, Dong et al. (2020) developed red-light-emitting carbon dots (R-CDs) combined with starch (Dong et al., 2020). In their study, R-CDs were synthesized and combined with starch to create a chemical powder that effectively illuminated LFPs on various surfaces, providing clearer visibility and enhanced identification accuracy. This innovative approach used an AI-assisted digital processing program to analyze the fluorescence images of the enhanced fingerprints. The program achieved a high match score of 93% when comparing processed LFPs to standard control samples, significantly outperforming traditional methods. The study highlighted the advantages of R-CD/starch phosphors, including their high photoluminescence properties, low cost, and ease of preparation, making them suitable for practical applications in forensic science.

Most fingerprint databases rely on the condition of the finger's surface, which can affect the accuracy of identification systems. Working with low-quality fingerprint databases presents significant challenges for researchers, complicating the identification process and affecting system accuracy. In this context, a fingerprint identification system utilizing ML techniques was developed to enhance fingerprint image quality, extract relevant features, and match the prints with a database (Nguyen et al., 2022). The identification process involves several key steps, including image preprocessing to enhance quality, feature extraction focusing on the unique characteristics of the fingerprint, and template matching in the database. The proposed method uses image processing techniques such as noise filtering, histogram equalization, and segmentation to overcome challenges posed by low-quality fingerprint images. The system achieved a high accuracy rate of 97.75% in a mixed-quality fingerprint database, demonstrating the effectiveness of neural networks in improving identification performance.

In addition to the successful applications of DL techniques in fingerprint classification, innovative methods such as DNN have significantly enhanced system accuracy. An

innovative approach to fingerprint classification using an Automatic Deep Neural Network (ADNN) model has been proposed (Mahmoud et al., 2023). This model autonomously determines key parameters, such as architecture, number of filters, epochs, and iterations, to achieve high accuracy. The proposed system employs a CNN and automates both the feature extraction and classification stages, significantly reducing the time and effort required for fingerprint recognition. The model achieved 99.75% accuracy on the SOCOFing dataset, outperforming existing models. As a result, the proposed ADNN model represents a major advancement in fingerprint recognition technology, offering a fully automated solution adaptable to different datasets and user inputs.

2.8. AI Applications in Forensic Ballistics

Forensic ballistics involves the scientific examination of ammunition recovered in cases involving firearms and other weapons, aiming to evaluate the connection between the weapon and the incident. This discipline helps identify the type, make, and model of the firearm and determine the shooting distance by analyzing bullet casings and cartridges collected from the crime scene (Heard, 2011). In addition to traditional methods, AI technologies have accelerated the process of identifying and comparing marks on bullets and cartridges, allowing databases to detect similarities and differences with high accuracy, thus making investigations more reliable (Kudonu et al., 2022).

Cartridges collected from crime scenes are often used to determine the type and model of the firearm. Experts in firearms and ballistic residue evaluation rely on collections of cartridges from various firearms, specialized databases, and reference sources to identify class characteristics. However, this process largely depends on the classifier's knowledge and experience. In a study investigating the use of ML for classifying firearm types and models from cartridge images, a database was created using the IBIS BrassTrax3D system, which ensures consistent resolution and quality of the images. The dataset consisted of 620 images from seven categories of semi-automatic pistols (Giverts et al., 2024). The results showed that the classification accuracy of ML models

ranged from 71% to 81%, and with an ensemble approach, the rate increased to 87.8%. Despite these positive results, the study emphasized the need to expand the training database to include more examples and different firearm categories. Additionally, adding an undefined category for cases where the model could not confidently classify a cartridge was recommended.

Bullet ricochet, a deviation that alters the initial trajectory and speed of a bullet, is common in both accidental and intentional shootings (Haag & Haag, 2020; Kerkhoff et al., 2015; Muster, 2020; Nishshanka et al., 2020, 2022). In reconstructing shooting incidents, ricochets can provide valuable information, but few studies have systematically examined the morphology of ricochet marks (Haag & Haag, 2020; Malik et al., 2022; Nordin et al., 2020). A study focused on analyzing the morphology of ricochet marks on concrete from five different types of bullets fired at two distances. The researchers analyzed 297 ricochet impact points, using morphological features such as length and perimeter-area ratio to classify bullet types using the RF ML algorithm (Eren et al., 2024). The findings indicated that the .22 LR bullet left the most distinctive marks, and when the distance variable was included, the classification accuracy improved from 62% to 66%. The research noted significant overlap in the morphology of ricochet marks between different bullet types, raising concerns about the reliability of human identification in real-world scenarios. The study highlighted an important step in understanding ricochet marks and emphasized the need for a more comprehensive approach to forensic analysis.

Another study exploring the applicability of ML techniques in predicting ballistic outcomes in terminal ballistics focused on the ballistic limit of armor and bullet penetration depth (Ryan et al., 2024). Using a dataset of approximately 1,000 samples, four regression models were evaluated: XGB, ANN, Support Vector Regression (SVR), and Gaussian Process Regression (GP). The results showed that all models performed well on training data but struggled to generalize beyond that range. Some models improved when combined with engineering features, though SVR and XGBoost experienced performance declines. The findings revealed that

while ML models can effectively predict ballistic outcomes, their performance depends on the quality and scope of training data. Careful tuning and validation are required to avoid overfitting and ensure reliability in practical applications.

2.9. AI Applications in Digital Forensics

Digital forensics is defined as the process of obtaining valuable information and evidence from computing devices within a legal framework, and it is widely used by law enforcement and various organizations (Al Fahdi et al., 2016; Casey, 2010). AI plays a significant role in digital forensics due to its ability to rapidly process large datasets, perform complex analyses, and utilize DL algorithms for detecting digital traces (Al Fahdi et al., 2016).

Digital forensics faces challenges such as growing data storage capacities, the widespread use of flash drives, and the need to analyze numerous devices (Casey & Stellatos, 2008; Garfinkel, 2010). While many forensic tools offer "Push-Button Forensics" functionality that automates basic procedures, manual and cognitive evaluation is still necessary for data analysis. This places a burden on investigators, causing law enforcement to shift from a gold-standard approach to an intelligence-based one (Al Fahdi et al., 2016; Lawton et al., 2014). In this context, researchers aimed to reduce the time spent analyzing irrelevant data by using the Self-Organizing Map (SOM) technique to automatically group important files (Al Fahdi et al., 2016). The study found that this method effectively grouped data and achieved a recall rate of over 93% in identifying irrelevant files. Additionally, the study introduced the Automated Evidence Profiler (AEP) tool, which identifies and prioritizes irrelevant clusters based on the nature of the crime and metadata associated with the files. Using file interaction timelines, AEP aims to maximize the identification of relevant evidence while minimizing the analysis of noise files. The results showed that AEP provided promising recall rates, indicating that such approaches could facilitate digital forensic investigations. A study by Aditya et al. (2018) employed advanced technologies like big data, cloud services, and DL techniques to address the complexities of digital forensic investigations (Aditya

et al., 2018). The study noted that the resilience of DNN models in forensic contexts had not been thoroughly explored, so the authors proposed an Adversarial Testing Framework (ATF) to systematically evaluate the security resilience of black-box DNNs, particularly in forensic applications. The ATF aimed to solve two key challenges: generating inputs that reveal erroneous behavior in DNNs and adapting black-box attacks to various threat models. The framework included components like the Threat Model Adapter, Feature Impact Calculator, and Sensitivity Tester, which work together to generate attack samples and assess DNN defenses. To demonstrate the effectiveness of ATF, the authors conducted a case study using a commercial DNN service, Image Analyzer. The results showed that ATF significantly outperformed existing attack methods, highlighting the potential to improve the reliability of DNN applications in digital forensics.

The rise in cybercrimes targeting children through online platforms has significantly increased the need for digital forensic investigations (Anderson et al., 2019). Traditional methods used by forensic experts have become more challenging due to the growing volume and complexity of data (Hitchcock et al., 2016). Online sexual exploitation, a crime in which offenders use social media to target minors, is a prominent example of these challenges. To overcome these issues, technologies like ML have been proposed. These technologies can enhance the efficiency of investigations by automating the analysis of large datasets, such as chat logs, and assist law enforcement in identifying and tracking offenders. Ngejane et al. (2021) proposed integrating ML techniques into the Digital Forensic Process Model (DFPM) for detecting and classifying abusive language in chat logs (Ngejane et al., 2021). The study trained various ML models using the PAN-12 dataset and examined the models' ability to identify keywords and phrases associated with abusive behavior. Furthermore, the study aimed to provide forensic experts with tools to enhance the speed and accuracy of their work. Future research should focus on collecting more diverse datasets to improve the real-world effectiveness of these models.

Detecting manipulated multimedia content using ML has become another critical area of research

in recent years. This development is driven by growing concerns over fake news and misinformation, the need for effective forensic analysis tools, and the potential impact on national security (Ferreira et al., 2021). Researchers developed a system using CNN to detect manipulated images, videos, and audio (Anvekar et al., 2024). The method involves preprocessing data, extracting features, and training the model using large datasets composed of both real and manipulated content. The researchers emphasized the importance of techniques like Error Level Analysis (ELA) for images and Mel-Frequency Cepstral Coefficients (MFCC) for audio to enhance the detection capacity of CNN. The study also highlighted challenges, such as the need for high-quality datasets and adversarial attacks that could impact the effectiveness of detection models. In conclusion, the study presented a promising multimedia framework capable of distinguishing between real and manipulated content, demonstrating the successful application of the proposed system in real-world scenarios.

3. Risks, Ethical, and Legal Dimensions of AI Use in Forensic Science

Forensic science applies a range of scientific techniques and methods to clarify criminal cases, apprehend perpetrators, and protect the rights of the innocent (Suzanne, 2019). AI technologies, which reduce, improve, or accelerate these processes, have become critical in forensic science. The use of AI in various fields of forensics, such as forensic genetics, forensic medicine, and crime scene investigation, enables the rapid and efficient processing of large datasets, extraction of meaningful information, cost reduction, increased efficiency, and minimization of human error (Ahmed Alaa El-Din, 2022; Gupta et al., 2020; Singh Sankhla et al., 2020). However, integrating AI into forensic science has introduced several ethical and legal concerns.

AI technology in the criminal justice system is still underdeveloped. When evaluating AI use, law enforcement agencies must consider basic human rights principles such as privacy and non-discrimination, and whether AI algorithms can be as objective and intelligent as human

judgment. Since humans create AI algorithms, they may reflect human errors. Even though AI systems can operate independently of human intervention, they are still subject to potential inaccuracies because people develop them. AI-based algorithms rely on datasets derived from human data, and biases within these data can influence the algorithms' results. This raises serious questions about AI's role in justice (Babuta & Oswald, 2019; Muller, 2020). Additionally, independent studies have shown that AI use could lead to certain groups being disproportionately targeted by law enforcement. This could undermine principles of justice and equality (Raso et al., 2018). In particular, AI surveillance in specific regions could increase geographic discrimination, leading to higher arrest rates in those areas. Furthermore, the fact that private companies often provide the databases used by law enforcement poses various risks regarding data security and privacy. Misuse or exposure of these databases to cyberattacks could violate many citizens' privacy rights.

There are several ethical dimensions to the use of AI in forensic science. Since forensic analyses often involve personal data, protecting this information is of paramount importance. The unauthorized sharing or leaking of such data could violate individuals' privacy rights and lead to negative consequences (Oliva et al., 2022). Moreover, the accuracy of AI analysis depends on the database size used. If the database is not sufficiently large, AI algorithms may produce false positives or false negatives, which could lead to the reflection of human errors in the algorithms and reduce the reliability of the results (Ahmed Alaa El-Din, 2022). Research on the potential weaknesses of algorithms and their effects on forensic processes highlights the strengths and weaknesses of specific methods. For instance, while probabilistic genotyping offers high accuracy in DNA analysis, it also raises concerns regarding transparency and validity. In this context, courts' understanding, and evaluation of algorithmic processes are crucial to ensuring the right to a fair trial (Richmond, 2020).

Conclusion

In recent years, AI has been integrated into forensic science, adding a new dimension to identification processes. This integrated approach highlights the use of AI algorithms as a significant innovation in the field of forensic science. AI algorithms accelerate the identification process by supporting and automating decision-making in evidence identification. Applying AI tools in forensic science establishes a scientific foundation that reduces human error and enhances the strength of evidence subject to subjective evaluation. Furthermore, by modeling the cognitive abilities of human skills through mathematical methods, AI strengthens the scientific foundation of forensic methods and enhances the professional competencies of experts. This allows for presenting alternative perspectives in court cases, thereby improving the accuracy and reliability of forensic examinations. However, the role of AI in forensic analysis must be carefully considered from ethical and legal perspectives. This is a critical step to ensuring justice and securely integrating AI technologies into forensic science.

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A NEW TOOL IN FORENSIC SCIENCE: WASTEWATER-BASED EPIDEMIOLOGY

Aslı ATASOY AYDIN

Chapter 2

A New Tool in Forensic Science: Wastewater-Based Epidemiology

ASLI ATASOY AYDIN[1]

Introduction

Waste analysis is a resource frequently used by law enforcement agencies, not only in criminal investigations but also to understand criminal and illegal activities better. Like Edmond Locard's exchange principle in 1920, which says, "Every contact leaves a trace," waste is a trace (Edmond, 1920). Waste, which can be considered as the by-product of an activity, has the potential to provide important information about the occurrence and evolution of events when analyzed from the perspective of forensic sciences.

The analysis of waste is widely used in various fields of forensic science, particularly in forensic toxicology, to detect traces of alcohol, psychoactive substances or toxic substances in individuals' urine. In addition, waste is considered an important component of intelligence that provides information on illicit activities. For example, to locate clandestine laboratories that produce/synthesize illicit substances, one can investigate the areas where producers deposit their waste after the synthesis process. Analysis of these wastes can reveal the nature of the incident, that is, the type of illicit substances produced, while waste profiling can also be used to correlate different waste sites.

As physical remains or traces that provide objective and relevant information on a past incident, waste is considered important evidence that contributes to understanding specific events. This book chapter will focus on how waste is used as traces in the forensic process and discuss one of the different ways of using waste, the wastewater-based epidemiology (WBE) approach.

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1. Wastewater Based Epidemiology (WBE)

WBE is a methodology utilized to collect information or monitor the effects of human activities by analyzing wastewater content; the concept is not new. In this method, data on human activities, especially community-wide drug use and chemical exposure, are obtained by analyzing the substances carried in wastewater. WBE is a well-established analytical technique used in public health and environmental sciences that can provide comprehensive information on public behavior by monitoring chemical and biological markers (Daughton, 2001a). For more than two decades, it has become a large-scale research area, with scientists from different disciplines analyzing wastewater to understand the behavior of a community. In the beginning, the focus was on the effects of therapeutic drugs on humans in surface water sources, including rivers and lakes (Calamari et al., 2003). This approach quickly focused on the analysis of illicit and other substances (Zuccato et al., 2005). The substances consumed by the population connected to a specific sewage treatment plant were monitored using this approach. Over time, this methodology has been acknowledged as an additional epidemiological indicator, offering near real-time and objective results that contribute to a comprehensive discerning of the situation under investigation (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2016). However, despite the advantages of this approach for the intake of illicit drugs, the use of waste also has some limitations.

It is a complex matrix for detecting various chemical and biological markers, including wastewater, therapeutic and psychoactive drugs, tobacco, and alcohol (Choi et al., 2018). WBE offers an innovative analytical strategy for monitoring substance use and/or exposure in a population by measuring levels of these markers (Gracia-Lor et al., 2017). Chemicals consumed by humans are eliminated from the body via urine and feces, entering the wastewater system and being collected in sewage treatment plants is the basic principle of WBE (Figure 1). In addition to licit and illicit drugs, this method provides information on a wide range of human health issues, such as environmental pollutants, industrial and

agricultural chemicals, infectious diseases, pathogens and antibiotic resistance (González-Mariño et al., 2020; Rousis et al., 2020; Van Hal, 2019; Yavuz Guzel et al., 2021, 2024). This strategy has also been applied to monitor new psychoactive substances (NPS) and has become an important tool to evaluate substance use at a public level as a rapid and affordable method over the last few years (Atasoy Aydin et al., 2023).

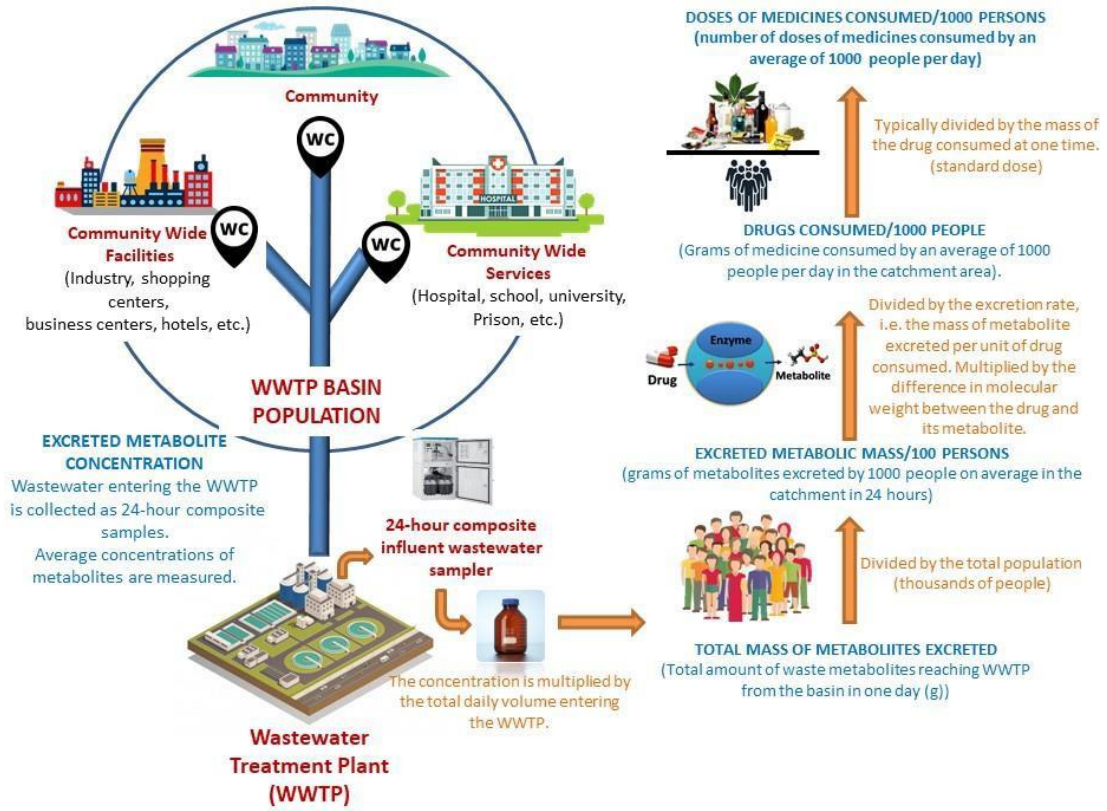

Figure 1: Schematic illustration of WBE workflow: the process of converting measured substances to daily mass load or per capita consumption *(Dağlıoğlu et al., 2024*)

In 2001, the WBE was first used to assess illicit substances and therapeutic drugs in the community (Daughton, 2001a). It was used in 2005 to assess cocaine use and track local drug trends in Italy (Zuccato et al., 2005). WBE has become an important tool for the assessment of the use of illicit drugs through the detection of drugs and their metabolites specific to human beings in wastewater. The first international study was conducted in 19

European cities in 2012 (Thomas et al., 2012). In 2013, a comprehensive study was presented addressing uncertainties in the WBE method (Castiglioni et al., 2013). Another study summarizing WBE studies between 2005-2017 on a global scale was studied (Feng et al., 2018). Recognized as a promising and novel method for detecting illicit drugs, WBE offers advanced methodologies for the measurement of a variety of illicit substances (Hernández et al., 2018, 2019).

Chemicals used or exposed by individuals are eliminated in unaltered form or as metabolites in urine or feces and reach the wastewater system or directly into surface waters in environments where there is no sewage system (Daughton, 2001a). If these substances remain stable in wastewater, the chemicals reaching the wastewater treatment plant (WWTP) in a certain period may reflect the substance use of the population served by WWTP during that period. Taking into account the pharmacokinetic properties of drugs and their environmental effects, drug consumption in a given community can be estimated using the amounts of these substances (Daglioglu et al., 2021; Zuccato et al., 2005, 2008). In this context, wastewater analysis offers a valuable analytical method for assessing the drug consumption of communities within a given sewerage network in a non-invasive, timely and anonymous manner, providing reliable data normalized to the population (Bijlsma et al., 2021; Daughton, 2001b).

2. Methodology of WBE

The underlying principle of this analysis is that human consumption of psychoactive drugs is excreted in urine or feces as metabolized products, consisting of a combination of unchanged substances and metabolites, leaving detectable traces. These traces can be detected and quantified because they are transported through the sewer system to the WWTP (Daughton, 2001b). Many scientists have focused on developing the scientific foundations of WBE and have proposed applications in various fields (Guzel et al., 2023; Yavuz Guzel et al., 2024; Zarei et al., 2020).

2.1. Collection of Wastewater Samples

One of the most critical elements of WBE studies is wastewater sampling. Sampling should be carefully planned and implemented in accordance with the research question. Sampling designs range from instantaneous samples taken in a matter of minutes to composite sampling methods, where many subsamples collected in proportion to time, flow, or volume are combined. Ort and colleagues have extensively explored this topic and proposed best practice methods to enhance the comparability of data from various locations. They emphasized that a composite sampling strategy proportional to the flow rate (preferably continuous) is the most appropriate for the collection of representative samples of wastewater (Ort et al., 2010). However, because this method is quite complex to implement, simpler discrete approaches are generally preferred. To account for possible changes in analyte concentrations and flow rates during sampling, high-frequency sampling was considered ideal (e.g. every 5-10 minutes). Generally, sampling is continued for 24 hours to detect the total mass load of the analytes encompassing the WWTP. Composite samples are usually collected using automated samplers.

Illicit drug consumption in the community was estimated by collecting representative compound samples from raw municipal wastewater. Wastewater samples are gathered over a 24-hour period, either flow- or time-dependent, as they enter a treatment plant. Samples were collected by combining small aliquots taken at regular intervals by an automated and computer-controlled device, ensuring that the samples were reproducible and representative. However, in some studies, grab samples and polar organic chemical integrative samplers were also utilized (Hass et al., 2011). These alternative methods are also considered different sample collection strategies that can be used under specific conditions.

2.2. Selection of Drug-Target Residues to be Analyzed in Wastewater Samples

The substances considered in the selection of target residues from illicit drugs are commonly used globally. To back-calculate the consumption

estimates, the general criterion for selecting target residues is to identify the metabolite of each active substance that is stable in wastewater and most abundant as a specific urine product. These "target residues" are the key components that should be monitored in wastewater analyses of each illicit drug and are used for back-calculation usage levels of the substances.

2.2.1. Cocaine

In humans, only a small fraction of the ingested dose of cocaine is eliminated unchanged in the urine, with the majority (99-91%) being eliminated as a metabolite known as benzoylecgonine (BE) (Castiglioni et al., 2008). In forensic toxicology, BE is measured in urine to detect cocaine use (Castiglioni et al., 2006). Therefore, only BE concentrations were used for the measurement of cocaine usage, although in many studies, both BE and cocaine were measured in wastewater. An advantage of estimating cocaine consumption based on the BE metabolite rather than the parent compound is that unused cocaine excreted directly into the wastewater can be excluded from the calculations. Alternatively, when back-calculating cocaine, the bconcentrations of the parent compound cocaine have been used in some studies (Bones et al., 2007).

2.2.2. Cannabinoids

The main active compound in the *Cannabis sativa* plant is Δ9-tetrahydrocannabinol (THC). Upon inhalation, THC is quickly absorbed in the lungs and metabolized in the liver, producing the active metabolite 11-hydroxy-THC. This metabolite is subsequently oxidized into several compounds, with 11-nor-9-carboxy-THC (THC-COOH) being the predominant metabolite often selected as the target analyte (Chayasirisobhon, 2021). THC-COOH, mainly in its conjugated form with glucuronic acid, is excreted mainly in urine and feces (Huestis et al., 1996). However, glucuronidase enzymes present in untreated wastewater readily hydrolyze these conjugates to their free acid form (D'Ascenzo et al., 2003). Specific studies on the stability of THC-COOH in wastewater have confirmed this (Castiglioni et al., 2006). Therefore, it is widely used as a biomarker for monitoring cannabinoid consumption.

2.2.3. Amphetamine type stimulants

Amphetamine is excreted unaltered in the urine after consumption, usually between 30-74% of the dose. It is also eliminated largely unaltered in the urine as methamphetamine (43% of the dose). Similarly, methamphetamine's cyclic derivative, methylenedioxymethamphetamine (MDMA), is excreted unaltered in urine (65% of the dose). It is partly excreted as methylenedioxyamphetamine (MDA) (7% of the dose). It is an amphetamine derivative with hallucinogenic properties and is usually excreted unchanged at high doses (Baselt, 2004). Therefore, because amphetamine-type stimulants are excreted unchanged in urine, the substance itself is often chosen as the analytical target in wastewater analysis.

2.2.4. Opioids

In the body, heroin undergoes rapid deacetylation in the liver to form morphine and 6-acetylmorphine (6-MAM). Therefore, morphine, a metabolite common to heroin, codeine, morphine, and 6-MAM, a specific metabolite of heroin (1-3% of the dose), is often the focus of wastewater analysis (Baselt, 2004). The excretion of morphine partly occurs as glucuronide conjugates. These are hydrolyzed back to morphine in raw sewage (D'Ascenzo et al., 2003). This process can affect morphine stability in sewage samples and should be considered in the analysis.

2.2.5. New psychoactive substances (NPS)

Despite some limitations, the potential of WBE for the determination of the use of a given substance is of particular importance in the fight against the increase in the quantity of 'new psychoactive substances' (NPS) in the illicit drug market. According to a 2023 report by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), over 950 new psychoactive substances (NPS) are being monitored by the end of the year. 26 of these substances were first notified in Europe in 2023. The report emphasizes that new synthetic opioids, such as synthetic cannabinoids, synthetic cathinones, and nitazenes, pose serious health risks. Nitazenes, in particular, present a crucial threat because of their high potency and have been associated with several cases of poisoning (European

Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2023). Their molecular structures still fall outside the scope of international agreements, including the 1961 UN Single Convention on Narcotic Drugs and the 1971 UN Convention on Psychotropic Substances. This legal loophole provides an advantage for producers and sellers who openly market their products online.

Countries have been warning about the potential dangers of these new psychoactive substances in their jurisdictions and requesting more information. However, incoming information on these substances is often limited to standard population surveys or existing harm reduction statistics. One reason for this limitation is the limited access to the population that has shown interest in these new substances, often referred to as 'psychonauts', people who want to try new substances and explore their effects. These individuals are usually not under treatment, so they are difficult to monitor and evaluate using traditional methods (Rossy et al., 2017).

The WBE methodology can make a valuable contribution to early warning systems through its potential for determining NPS use and understanding the patterns of use. In the field of public health, this method can play a crucial role in legal strategies by offering an effective approach to validate and monitor problematic situations related to these emerging substances.

2.3. Back Calculation

The WBE was calculated by normalizing the biomarker concentration in the influent water of wastewater treatment systems to per capita mass loads using the daily flow rate and the population served by the WWTP (Figure 1). The WBE allows per capita intake, release, or exposure of biological or chemical agents to be assessed at the community level through back-calculations. An important aspect of WBE is adequate and representative sampling of substances of concern in wastewater samples. The per capita daily consumption of a major compound in a wastewater collection basin was measured using the following formula (Formula 1) (Choi et al., 2018)

$$\text{Daily consumption}_i \text{ (mass/day/1000person)} = \frac{Cii * Vttttttttt * \frac{Rii}{Eii}}{N}$$

(Formula 1)

where C_i means the concentration of the target analyte (parent substance or metabolite) in influent wastewater samples, V_{total} means the total volume of wastewater entering the wastewater treatment plant during the sampling (usually 24-h), N means the total population served by the WWTP, E_i is the average excretion rate of a drug residue, and R_i means the ratio of the molar mass of the parent substance to its metabolite.

Correction factors used in back-calculations often consider a drug's metabolism and excretion (especially through urine). To determine the appropriate correction factors to be used in back-calculations, investigators should thoroughly review the available pharmacokinetic data and refine these factors (Castiglioni et al., 2014). The choice of metabolites rather than the main drug as biomarkers in wastewater analyses is also of great importance, as the choice of metabolites plays a crucial role in distinguishing whether a drug is present in wastewater due to active human use or as a direct result of destruction or synthesis processes. In particular, the use of metabolites provides a reliable method to determine whether the use of illicit substances is due to human consumption. This approach helps avoid biases related to residues discharged directly into wastewater or residues from chemical production, thus making consumption estimates more accurate (Daughton, 2001b). However, some uncertainties may arise in estimating drug consumption because factors like the "standard dose" and mode of intake (chronic, occasional or intensive use) can vary considerably. (van Nuijs et al., 2011). Therefore, pharmacokinetic data is required to obtain reliable estimates. One of the challenges in detecting drugs of abuse by wastewater analysis is the limited human toxicokinetic and pharmacokinetic data for traditional illicit drugs; for NPS, these data are even more scarce (Reid et al., 2014). Due to ethical and safety restrictions, pharmacokinetic studies for drugs of abuse are highly complex. They are not yet adequate for some substances

because they can only be conducted in authorized research centers (Castiglioni et al., 2014).

2.4. Chemical analysis

Analyzing target compounds in composite samples at very low levels (nanograms per liter or less) requires highly sensitive and selective techniques. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) or high-resolution mass spectrometry (HR-MS) are the most commonly used techniques for determination and quantification (Bijlsma et al., 2021). Before instrumental analysis, a critical step is the proper preparation of samples to minimize matrix effects and concentrate target analytes. This preparation step is mostly imposed by solid-phase extraction (SPE), where the target analytes are adsorbed onto an SPE, allowing samples to be concentrated 100-fold or more (O'Brien et al., 2014). However, recent advances in instrumental techniques, such as large injection volumes and 'dilute and draw', have enabled the use of time-saving alternative methods (Berset et al., 2010).

The toxicokinetics (how a drug is absorbed, distributed, metabolized, and excreted) of each target drug is a vital component in quantifying the amount of the parent drug or its metabolite discharged into the sewerage system (Daughton, 2001b). Several factors, including drug type, dose, route of administration, and personal factors (e.g., medical status and age), influence the toxicokinetic process. In addition, the gut microbiome could have a significant function in the biotransformation of ingested foreign substances, affecting the formation of metabolites that are excreted in the urinary and fecal systems and may enter the sewage system (Daughton, 2001b). In the process of estimating population-wide drug use, it is necessary to consider that potential biases may influence the accuracy of the results and minimize these uncertainties (McCall et al., 2016). The choice of target substances and further calculations should take into account the chemical properties of related metabolites and excretion rates of parent and metabolites, especially the parent/metabolite ratio in urine (Reid et al., 2014).

3. The Use of WBE in Forensic Science

Since the first application of WBE, a variety of studies reported the determination of different illicit substances in wastewater (Asicioglu et al., 2021; Daglioglu et al., 2021; Daughton, 2001a; Zuccato et al., 2005), researchers have also conducted studies in places such as music festivals and prisons to observe the impact of restricted populations on consumption patterns (Baker et al., 2014; Postigo et al., 2011). They also analyzed wastewater. Scientists and law enforcement agencies have recognized that information from the wastewater analysis of illicit drug residues has great potential, particularly for intelligence purposes. (Castiglioni et al., 2013).

From a forensic intelligence perspective, a clearer and more accurate assessment of the situation can be achieved by bringing together different data on illicit drug trafficking and drug usage (Bijlsma et al., 2021; Castiglioni et al., 2013). By combining data from different sources, more accurate hypotheses can be developed regarding the types of illicit substances available in the market and the substances used. The combined analysis of multiple data sources, including WBEs, enables law enforcement, policymakers, treatment providers, and harm-reduction programs to make informed decisions. In addition, changes in the types and quantities of illicit substances can be used to adapt prevention programs (Castiglioni et al., 2013).

Biomonitoring using the WBE approach has emerged as an alternative and complementary method for population drug use (Bannwarth et al., 2019). Combining data from wastewater analysis with the chemical profiles of seized illicit substances can be an effective strategy to provide more comprehensive information on illicit substance use and the market for these substances (Sodré et al., 2017). This method has been used at music festivals (Benaglia et al., 2020), holiday periods (Bade et al., 2021), sporting events (Montgomery et al., 2021), and most recently during COVID-19 (Reinstadler et al., 2021; Yavuz-Guzel et al., 2022). WBE has proven to be a useful tool for tracking changes in substance use during special events, including the use of alcohol (ethanol), tobacco, and illicit substances. In this regard, WBE has been extensively

studied to track spatial and temporal trends in tobacco, substance, and alcohol use (Yavuz Guzel et al., 2021). In addition, analysis of chemical profiles resulting from the discharge of waste from the production of illicit substances into the sewerage system can assist in the detection of clandestine laboratories (Emke et al., 2018).

WBE can make important contributions to forensic-intelligence strategies, such as monitoring and controlling substance use (Been et al., 2016). Analyses of illicit substances in wastewater are generally consistent with other epidemiological figures and complement information from seizure reports on illicit substances and population surveys (Castiglioni et al., 2013). In monitoring substances of abuse, WBE allows for rapid assessment of substance trends, measurement of the effectiveness of substance abuse programs and comparisons based on population surveys. Results from these analyses can be used to interpret the size and dynamics of illicit substance markets, allowing law enforcement to assess indirectly the impact of specific criminal groups on the distribution of illicit substances (Been et al., 2016).

In the context of forensic intelligence, a more comprehensive awareness of drug problems and strategic intervention and treatment plans can be achieved by combining the findings of wastewater analysis with other data sources (Bade et al., 2021; Benaglia et al., 2020; Sodré et al., 2017). In addition, data received during investigations and law enforcement operations can help reduce uncertainties arising from wastewater analyses. With the widespread use of NPSs, combining wastewater analysis with drug seizure reports and toxicological analysis is emerging as an effective strategy (Been et al., 2016). Wastewater analysis can also function as an early warning system to detect new substances of abuse in the drug market. However, detecting NPS requires toxicokinetic data, understanding the fate of chemicals in wastewater, and reference materials (Castiglioni et al., 2014).

3.1 Substances Analyzed in Wastewater from a Forensic Science Perspective

WBE has been used in forensic investigations since the mid-2000s. In 2005, the analysis of cocaine in wastewater was studied for the first time (Zuccato et al., 2005). Since then, many scientists have examined the presence of licit and illicit drugs in wastewater worldwide. Nicotine is widely consumed around the world and can be measured directly in wastewater. Nicotine can also be considered an indicator of human use and is an important biomarker for quality control and data normalization (Daughton, 2001b). Several studies have detected nicotine metabolites, including trans-3'-hydroxycotinine and cotinine, in wastewater samples to assess nicotine utilization (Aşıcıoglu et al., 2021; Reinstadler et al., 2021).

Ethanol is among the most widely consumed substances globally and can be effectively quantified using wastewater analysis. A small quantity is metabolized to ethyl sulphate (EtS) and ethyl glucuronide (EtG) (Helander & Beck, 2005). They are common ethanol metabolites found in the urine after the consumption of alcohol (Helander & Beck, 2005; van Wel et al., 2016). However, given the instability of EtG in wastewater, EtS is preferred as a more reliable biomarker to estimate ethanol consumption. The direct elimination from the body of alcoholic drinks, as well as other products, such as hand disinfectants, should also be considered as a source of ethanol in wastewater (Reid et al., 2011). This has been an important factor in wastewater investigations because of the widespread use of ethanol-containing hand disinfectants during COVID-19. However, the minimal effect of ethanol in sewage on EtS formation does not affect the choice of EtS as a biomarker (Reid et al., 2011). EtS in wastewater samples has been proposed to estimate ethanol consumption (Fallati et al., 2020; Yavuz Guzel et al., 2024).

Cannabis is reported by the United Nations Office on Drugs and Crime (UNODC) to be the most widely used illicit substance, with around 219 million users worldwide in 2023 (United Nations Office on Drugs and Crime (UNODC), 2023). According to UNODC, trends in cannabis use are changing as a result of being legal in some countries, and it will take some time to evaluate the effects of legalization on non-

medical use fully, so careful monitoring of the cannabis market is needed (United Nations Office on Drugs and Crime (UNODC), 2023). One of the most widely used biomarkers for the estimation of cannabis use in WBE applications is THC-COOH, the main active ingredient in cannabis (Pandopulos et al., 2020). THC-COOH is generally detected at higher concentrations in wastewater and has been detected in wastewater in a number of studies (Benaglia et al., 2020; Fallati et al., 2020; Yavuz Guzel et al., 2024). It has also been reported in the literature that THC itself has been determined in wastewater, and THC-OH, another metabolite of THC, has been published in some studies (Croft et al., 2020; Gago-Ferrero et al., 2020). It should be taken into account that THC metabolites can be eliminated through feces, adsorbed and deposited on particulate matter in wastewater due to their lipophilic properties (Pandopulos et al., 2020). Cannabidiol (CBD), another important cannabinoid found in cannabis, is a non-psychoactive compound that has therapeutic properties and is usually excreted in its main form through urine (Apul et al., 2020). A recent study analyzed CBD and its metabolites CBD-7-COOH and CBD-7-OH in wastewater, detecting only CBD (Pandopulos et al., 2020). As cannabis is increasingly legalized for medical and recreational use, THC-COOH concentrations in environmental waters are likely to increase over time (Apul et al., 2020). Other cannabinoids found in cannabis have also shown this trend.

The stimulant class is a frequently detected and reported class of illicit substances in wastewater. Detection of cocaine and/or BE in wastewater samples is common (Asicioglu et al., 2021; Dağlıoğlu et al., 2021; Reinstadler et al., 2021; Rice et al., 2020). Some studies have published other cocaine metabolites, including ecgonine methyl ester (EME) (Gago-Ferrero et al., 2020), norcocaine (Montgomery et al., 2021), anhydroecgonine methyl ester (AME) (Rice et al., 2020), and cocaethylene (Croft et al., 2020; Montgomery et al., 2021). Amphetamine and/or methamphetamine are frequently detected in wastewater analysis (Benaglia et al., 2020; Elder et al., 2021; Fallati et al., 2020; Gago-Ferrero et al., 2020; González-Mariño et al., 2020). However, because these

substances can be metabolites of other drugs, including fenproporex, selegiline, and famprofazone, wastewater may overestimate amphetamine or methamphetamine use (van Nuijs et al., 2011). MDA, MDMA, and other amphetamine-like drugs are frequently detected in wastewater samples (Asicioglu et al., 2021; Benaglia et al., 2020; Dağlıoğlu et al., 2021; Fallati et al., 2020; Gago-Ferrero et al., 2020; Kasprzyk-Hordern et al., 2021; Reinstadler et al., 2021). HMMA and HMA, metabolites of MDMA, have been found in wastewater samples (Rice et al., 2020). Some researchers have suggested the inclusion of HMMA and HMA metabolites in analytical methods for estimating MDMA use (González-Mariño et al., 2017). The chirality of the MDMA and MDA is important. The sources of MDMA (direct use or disposal) and MDA (MDMA metabolism or direct use) in wastewater can be better understood using chiral analytical methods (Kasprzyk-Hordern & Baker, 2012).

Opioids are substances used to treat moderate to severe pain. This class includes prescription painkillers such as oxycodone, hydrocodone, codeine and morphine. They also include synthetic opioids such as heroin (an illegal drug) and fentanyl (Sarhill et al., 2001). Opioids frequently detected in wastewater include morphine, codeine, methadone and its metabolite EDDP (Croft et al., 2020). These compounds are recognized as the main sources of opioids in surface waters (Campos-Mañas et al., 2018). Heroin has also been detected in wastewater in some studies (Benaglia et al., 2020; Croft et al., 2020). 6-MAM, a unique metabolite of heroin, may not have been widely reported in wastewater samples because of its low concentration. However, 6-MAM was also detected in other studies (Croft et al., 2020; González-Mariño et al., 2020). Difficulties in estimating heroin use from morphine concentrations in sewage analysis, as morphine may come from both therapeutic and illegal use (van Nuijs et al., 2011). The therapeutic use of morphine and the amount of morphine resulting from codeine metabolism should also be taken into account when assessing heroin use on the basis of morphine levels (Rice et al., 2020). It is also important to note that the consumption of poppy seeds can lead to the formation and elimination of morphine

in the urine (Özbunar et al., 2019; Smith et al., 2014).

Benzodiazepines are a group of drugs covering a variety of medicines, including diazepam, oxazepam, temazepam, alprazolam (Mandrioli et al., 2008). These drugs are commonly prescribed to treat conditions that include anxiety, insomnia and seizures. They are also used for their sedative, amnesic, and muscle-relaxant effects (Mandrioli et al., 2008; Temte et al., 2019). However, abuse of benzodiazepines has also been widely reported (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2018). Diazepam, a benzodiazepine, has a complex metabolic process and is metabolized together with its active metabolites, nordiazepam and temazepam. These compounds can be converted to other active compounds, such as oxazepam, which is glucuronidated and excreted as oxazepam-glucuronide. Glucuronidation is the main pathway involved in drug metabolism (Mandrioli et al., 2011). In wastewater analysis, diazepam and nordiazepam have been reported. Some studies have also found oxazepam and temazepam in wastewater (Gago-Ferrero et al., 2020; Reinstadler et al., 2021; Rice et al., 2020). Although zolpidem is not a benzodiazepine, its mechanism of action is like that of benzodiazepines. It belongs to the class of hypnotics known as "Z-drugs" (Mandrioli et al., 2011). Zolpidem is a hypnotic drug used for therapeutic purposes, but it has been linked to cases of abuse, addiction, and even fatal poisoning (Schifano et al., 2019). Zolpidem and its metabolite zolpidem-4-phenylcarboxylic acid have been found in wastewater in several literature studies (Schifano et al., 2019).

Wastewater analyses have also reported other substances involved in forensic cases. One of these is lysergic acid diethylamide (LSD), a semisynthetic hallucinogen derived from the ergot mushroom Claviceps purpurea. Recent studies have identified LSD and its metabolite, 2-oxo-3-OH-LSD, in wastewater (Bírošová et al., 2020). Ketamine is a phencyclidine (PCP) derivative, that exhibits anesthetic, analgesic, hypnotic, and amnesic properties, and its ability to induce dissociative anesthesia is well known. However, recreational and illicit use of ketamine is also common. Clinical and veterinary prescriptions can

contribute to the release of ketamine into the environment, as can illicit use (Lin et al., 2014). Another substance found in wastewater is ketamine and its metabolite norketamine (Bírošová et al., 2020; Rice et al., 2020). Gamma-hydroxybutyrate (GHB) is a chemical produced endogenously by the body through the metabolism of gamma-aminobutyrate (GABA). However, since the 1990s, GHB has been used as an illicit drug of abuse. It is also consumed as a dietary supplement and sleep aid (Busardò & Kyriakou, 2015). In drug-facilitated crime (DFC), it has also been used as a chemical agent (Odujebe et al., 2007). A recent study detected GHB in wastewater and concluded that the GHB detected was probably due to endogenous metabolism (Diamanti et al., 2019).

4. Advantages and Limitations of WBE

Wastewater analysis has several advantages over other epidemiological methods. These advantages include more objective estimates, lower costs, protection of individuals' anonymity and privacy, and near real-time monitoring of substance use without the need to collect biological samples from individuals. (Castiglioni et al., 2013; Daughton, 2001b; Sodré et al., 2017). Medical records, population surveys, and crime statistics are currently the official methods for estimating the use of illicit substances. However, because illicit substance use is socially stigmatized, these methods rely on honest reporting by users, which underestimates the extent of illicit substance use. In addition, rapid detection of changing trends in drug use through population surveys is time-consuming. Therefore, international drug agencies recommend the use of new methods that will provide more reliable information on this phenomenon (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2023).

Analyzing wastewater also involves some uncertainties that should be considered during the analysis. Substances reaching wastewater can originate not only from human excreta but also directly from the disposal of illicit drugs or synthetic products (Daughton, 2001b). For instance, medicines in powder, tablet, or herb form can often be disposed of in the sewerage system in a ready-to-use form, dissolved in wastewater, and reaching wastewater treatment plants.

This should be taken into account, and it is recommended that the human metabolism products of these substances be included in the methods used to avoid biases (van Nuijs et al., 2011). Nevertheless, performing metabolic studies for some new synthetic drugs may be necessary. Furthermore, techniques including enantiomeric profiling of chiral compounds can be used to determine sources such as illicit use, metabolism, or direct disposal as a waste of substances (e.g. amphetamine) (Kasprzyk-Hordern & Baker, 2012; van Nuijs et al., 2011).

WBE is a crucial addition to existing methods for monitoring illicit substance use. It is used to track trends in illicit substance use in many countries, understand the structure of substance use, and create maps of substance use an the international level (Castiglioni et al., 2014; Daughton, 2001b; Zuccato et al., 2005). Furthermore, WBE results were included in the panel of drug indicators used by EMCDDA. These results are extremely important, as they allow for a quantitative assessment of drug use at local, regional, and even national levels (van Nuijs et al., 2011).

When analyzing wastewater, there are a few factors to consider. The characteristics and conditions of the sewerage network and WWTP must be considered in WBE studies (van Nuijs et al., 2011). Variables such as the daily flow rate, the concentration of dissolved oxygen, the pH value, the existence of sediment, and the temperature can all influence the composition and status of the effluent, which in turn can have a direct effect on the stability of the medicines (McCall et al., 2016). For the accuracy of the calculations, data such as the whole population served by the treatment plant and wastewater flow rate are necessary. However, assessing the population covered by a given WWTP is a challenging process, as population changes can occur seasonally. Thus, there are two different approaches to determining the population served by a collecting system and treatment work: estimates based on chemical markers or a combination of census and collecting system capacity data (Castiglioni et al., 2014). However, seasonal variations in population may not be fully reflected in census and sewer capacity data (Been et al., 2014). Water quality factors, including biological and chemical

oxygen demand, total phosphorus, and nitrogen, may be used for population estimation, but they need to be considered carefully because they are subject to non-human influences. Another recently recommended marker is the ammonium ion, which has lower sensitivity to non-human sources (Been et al., 2014). However, further biomarker research is needed to identify the population served by treatment plants and monitor possible variations (Castiglioni et al., 2014).

Another important factor, especially when analyzing treated effluents, is that the processes that take place in wastewater treatment plants can affect the fate of drugs and metabolites present in the effluent. Traditional wastewater treatment plants target removing pathogens and E.coli bacteria from wastewater and reducing carbon, nitrogen and phosphorus loadings (Loos et al., 2013). However, in the natural environment, transformation products may be formed during chemical treatment processes in WWTPs and during hydrolysis or photolysis processes (Bletsou et al., 2015). For instance, one study examined the photolysis effects of cocaine and its metabolites when exposed to simulated sunlight or UV radiation and found that several transformation products were formed. The two products identified in the photolysis experiments, cocaine and benzoylecgonine, were also found in influent and effluent samples, although wastewater is not normally directly exposed to UV radiation or sunlight. The researchers concluded that some of these products may result from elimination from the body, while others may result from processes such as bacterial biotransformation occurring in sewage (Bijlsma et al., 2013). Another study investigated the effects of hydrolysis, photolysis and chlorination on THC-COOH. The transformation products of THC-COOH were not identified in the wastewater samples. However, hydrolytic and photolytic degradation products were observed in both wastewater and surface water samples. Certain WWTPs use progressive treatment methods, including ozonation, chlorination and UV radiation, which may lead to physical and chemical changes in drugs and metabolites (Boix et al., 2014).

The WBE is an advantageous tool for measuring illicit substance consumption and monitoring illicit

substance use in populations, but it also comes with several uncertainties and challenges. Despite these challenges, WBE is an important tool to supplement traditional epidemiological methods such as surveys, medical records and crime statistics. Although it cannot replace individual-level biomonitoring, WBE provides a cost-effective method for monitoring substance use in large populations by analyzing pooled urine samples (Aghaei et al., 2023). When used in combination with other data sources, WBE can provide a deeper understanding of drug consumption trends, making it a valuable asset in forensic science and public health contexts.

Conclusion

The analysis of substances in wastewater is considered a very promising tool for forensic science. It allows for monitoring substance use in a community and assessing illicit substance trends in a rapid and non-invasive manner. Such analyses provide important information that can support numerous public health and safety strategies. Data from WWTPs can be a particularly valuable resource for monitoring substance use. Many reports in the literature strongly support the identification of classical and new drugs of abuse in wastewater.

WBE is rapidly emerging as a critical tool in forensic science and investigations of forensic cases. It offers a broad view of crime and is a powerful source of data for detecting elements such as drug use and environmental threats at the community level. The role and importance of WBE in forensic science will only increase as technology advances and methodology improves (Daughton, 2018).

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EVOLUTION OF FORENSIC MICROBIOLOGY: FROM CLASSICAL METHODS TO DEEP LEARNING

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Chapter 3

Evolution Of Forensic Microbiology: From Classical Methods To Deep Learning

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Classical Methods

In many cases, microorganisms are ideally suited as physical evidence. Microorganisms live almost everywhere and can be found in human living spaces. Microorganisms can be collected from any place where forensic investigations are conducted, but not all microorganisms can be found everywhere. Another important feature of microorganisms is that they can undergo changes to form very durable structures in order to survive in adverse conditions. Considering all these features, it is not surprising that microorganisms have been used as physical evidence since the early days of forensic science, especially in determining the cause and time of death (Budowle et al., 2011).

Forensic microbiological examinations can enable the identification of the criminal through the characterization of microorganisms that are invisible to the naked eye but always present in the environment, in cases where the most important evidence of the crime scene, such as blood, DNA and fingerprints, are not sufficient to solve the crime. In forensic sciences, theoretical and practical knowledge of microbiology is generally used to present the microorganisms involved in the crime, their origins and potential effects as legal evidence. The field of study of forensic microbiology can be grouped under two main headings. The first type of study involves the direct use of microorganisms, examining and evaluating cases of biological terrorism, crimes, accidents, and evidence resulting from the unintentional release of microbial toxins. The second is studies where microorganisms are indirectly involved in the crime; poisonings caused by microorganisms multiplying in animal and plant foods or ingesting their secreted toxins, and

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practices considered crimes related to food. All examinations under these two headings include antemortem or postmortem investigations. In addition, forensic microbiology is used in postmortem examinations to prevent infection from being transmitted to the examining team from bodies that may carry infectious diseases. While classical evidence studies include steps such as crime scene investigation, a chain of custody applications, evidence collection, transportation, preservation, evidence examination, interpretation of the results and presentation to the court, forensic microbiological examinations, in addition to these, studies are also carried out to determine the etiology and type of the agent (Carter et al., 2017).

Forensic microbiology initially included applications in biocrime, bioterrorism, and epidemiology, but today, new approaches, such as the use of microorganisms as collateral evidence in criminal cases, to clarify causes of death (drowning, toxicology, hospital-acquired infections, sudden infant death, and shaking), to aid in human identification (skin, hair, and body fluid microbiomes), to determine a geographic location (soil microbiome), and to predict post-mortem changes (thanatomicrobiome and epinecrotic microbial community) are emerging (Oliveira and Amorim, 2018). It is important to reiterate that relying on a single piece of evidence is not acceptable when searching for a criminal; this is no different in forensic microbiology. Microbial evidence collected from a crime scene should be used in conjunction with other pieces to prove beyond reasonable doubt when a forensic case occurs (Blondeau et al., 2019).

The success of the analyses to be performed on microbiological evidence depends on the type of samples collected from the crime scene and how they are preserved. Depending on the characteristics of the evidence, the technique used to collect and record the evidence, the amount of evidence, the way to keep the evidence under control, the way to package the evidence and how the evidence should be preserved are determined. Microbiological evidence is collected within the scope of biological evidence. However, if a microbial examination is to be considered in evidence, great attention should be paid to the problem of

contamination (contamination). Any improper intervention (such as bare-handed contact, sneezing, coughing, or packaging errors) by individuals not involved in the incident can lead to contamination or degradation of the evidence, potentially rendering it unusable (Budowle et al., 2011).

There is no difference between clinical and postmortem techniques in sample collection. The type of samples to be taken postmortem should be decided by considering the premortem history of the case. Since the area opened during the autopsy is quickly contaminated by various means, the relevant organ surface should be sterilized during sample collection. There are two ways after the samples are taken and brought to the laboratory without wasting time. The first way to develop microorganisms is by culturing the samples taken. The second method includes metagenomic analyses based on the isolation of genetic material directly from the microbiological content of the samples. In culture methods, once the samples are collected, they should be incubated under varying conditions (such as temperature, pH, and oxygen requirements) and grown on different media to cultivate the potential microorganisms present, depending on their specific characteristics (James & Nordby, 2003).

In culture-based methods, microorganisms are isolated from their environment and studied by growing them in laboratory nutrient media. Different microorganisms may have special nutrient requirements and grow if these nutrient sources are available in the environment where they will be cultured. With classical methods, specific microbial species can be identified by culturing the microorganism in selective culture media, or they can be determined by staining methods such as Gram staining for physiological properties, morphological properties, growth characteristics in different nutrient media, and metabolite properties produced or used by the organism (Gürsoy and Otlu, 2017).

Identification involves determining the genus and species of a pure culture of a microorganism isolated from its environment by analyzing its cultural, morphological, metabolic (biochemical), serological, genetic, and other characteristics. When a microorganism is identified, it is placed in its place

within a previously determined identification scheme. Microorganisms are identified for practical purposes, such as helping to identify the criminal in forensic cases. Most identification procedures can be easily performed using several different tests. Protozoa, some algae, and fungi can be identified microscopically according to their morphological characteristics. Prokaryotic microorganisms generally exhibit morphologically similar characteristics and do not show much difference in shape or size. For this reason, many methods have been developed for the identification of prokaryotes. The tests used in the identification of microorganisms must be fast and reliable. A swab sample or any object may contain many microorganisms with very different characteristics. Therefore, microorganisms must be purified and turned into pure cultures before identification (Madigan et al., 2012).

There have been major changes in bacterial and fungal taxonomy in recent years. New methods have been introduced for species identification, and a mixed approach has been adopted in which phenotypic and genotypic analyses are used together to obtain more accurate results. Here, phenotypic analyses are performed to examine morphological, metabolic, physiological and chemical properties; genotypic analyses of cells are performed at the genome level. A successful identification is made at the species level by evaluating the results obtained. The identification system made in this way is called polyphasic taxonomy (Yılmaz-Cankılıç, 2009).

In morphological methods, the growth of the microorganism colony in the medium results in its unique morphology, odor, and metabolic properties. Although these properties give us little information about the microorganism, it is possible to determine the type of microorganism by looking at them. In addition, the examination of microscope preparations from stained and unstained microbial cultures also gives an idea about microorganisms. This method is very practical, but it has disadvantages in terms of the need for experience gained over the years and the high probability of making mistakes. Differential staining is often used to identify bacteria. This method is a microscopic method performed by using more than one dye.

These staining methods benefit from the chemical structure differences of the cell wall. Gram staining is the most well-known differential staining used in diagnosis. Although this type of staining cannot clearly determine the species, it groups bacteria as Gram (+) and Gram (–) (Madigan et al., 2012; Slonczewski and Foster, 2022; Yılmaz-Cankılıç, 2009).

Enzymatic activity determination, which is one of the biochemical tests, is used in identification because it varies among bacteria. Based on this situation, rapid biochemical tests that can be purchased as kits for fungi and bacteria have been developed. Rapid identification kits and devices have been designed for medically important bacterial groups. These systems perform many biochemical tests simultaneously and provide results within 4-24 hours. API and VITEK automatic test systems are good examples of rapid biochemical identification (Madigan et al., 2012; Yılmaz-Cankılıç, 2009).

Microorganisms cause the formation of antibodies when they enter the human body. Accordingly, microorganisms are identified by specific antigen and antibody screening.

Most microorganisms can be identified quickly by using serological tests. Examples include ELISA (Enzyme Linked Immunosorbent Assay), RAI (Radioimmun) and FAT (Fluorescent Antibody Test). ELISA tests are tests used in the screening of many diseases, the most important of which is AIDS. Fatty acid methyl ester (FAME) analyses are a method used mostly for bacteria in clinical and public health laboratories. Bacteria synthesize many different types of fatty acids, and these types differ among species. Commercial systems that separate cellular fatty acids perform identification by comparing known fatty acid profiles with the fatty acid profile of the analyzed bacteria (Madigan et al., 2012; Yılmaz-Cankılıç, 2009). The flow cytometry identification method is used to identify bacteria without culturing them. Identification with this method is achieved by collecting the signals given by the microorganisms in the sample illuminated by laser light in the flow cytometry device as they pass in front of the light and detecting them with the help of a detector (Yılmaz-Cankılıç, 2009).

Although microbial identification with developed culture methods is extremely cheap, it provides us with limited information about microbial diversity because they are time-consuming methods, and only a small part of the total variety of microorganisms that exist in nature can be grown in a laboratory environment (Gürsoy and Otlu, 2017). In recent years, advances in science and technology—such as molecular biological methods, parallel sequencing techniques, bioinformatics technologies, human identification, body fluid characterization, and the monitoring of infectious agents—have enabled forensic scientists to analyze even very small samples.

The Ascent of Molecular Techniques

Forensic microbiology involves investigating causes of death and criminal identification, geolocation and estimation of postmortem interval, much like other areas of forensic science. Along with other forensic sciences, it became internationally known and significant after a bioterrorism attack. In this attack carried out through the US postal service in 2001, letters containing *Bacillus anthracis* spores were used. In these attacks, 11 people suffered from inhalation anthrax, 11 people suffered from cutaneous anthrax, and 5 people lost their lives due to inhalation anthrax. In the investigation known as Amerithrax, the laboratory strain of the bacteria - *B. anthracis* Ames strain- was determined by multilocus variable number tandem repeat analysis. The molecular analysis led the investigation to the government biodefense laboratory and a scientist named Bruce Ivins. He committed suicide while the investigation was ongoing. In the years that followed, whole genome analyses revealed that the genotypes obtained did not even match the variants in the environments related to the investigation, only matching the *B. anthracis* types in the letters (Rasko et al., 2011; Lehman, 2012). This investigation is critical to proving the value of whole genome analysis of microorganisms in forensic microbiology.

With the development of major molecular biology techniques such as PCR and DNA sequencing, the Human

Microbiome Project (HMP) was launched. This has revealed that the microorganisms colonizing the human body are 10 times more than our cells. In addition, it has been shown that microbial diversity and quantity differ in different parts of the body and in healthy individuals (Ventura Spagnolo et al., 2019).

The conventional techniques in microbiology rely on culture, isolating colonies and identifying them through biochemical examinations. As a result, the culture conditions have an impact on the microbial community composition that can be obtained using these methods. The development of molecular techniques has led to the adoption of much quicker techniques like PCR for identification. Multiplex PCR, which allows the simultaneous use of different primer sets, or real-time PCR, which allows the results to be obtained simultaneously and consistently, are some of these techniques. The product obtained by PCR is sequenced using the Sanger method and compared with the data in DNA libraries. The markers commonly used in microbial identification are 16S and 18S rRNA. Unlike traditional methods, fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE), which do not require the cultivation of microorganisms, are important molecular methods that detect specific 16S rRNA and enable the identification of uncultivable microorganisms (Yuan et al., 2023).

A novel approach to molecular analysis is the Sanger sequencing method, which was previously discussed. The Human Genome Project was launched thanks to this method developed in the 1970s, and this large-scale project was completed using this technology. It is also known as the first-generation sequencing method. This method performs sequencing based on synthesis. In capillary electrophoresis, DNA fragments are detected with fluorescently labeled chain terminator nucleotides (ddNTPs). Today, the low throughput of Sanger sequencing is insufficient for complex genome analyses. In addition, its high cost and difficulties in application are other disadvantages (Yuan et al., 2023; Minogue et al., 2019).

Next-generation sequencing (NGS) technologies, capable of sequencing

millions of DNA at once, have also begun to be used in forensic microbiology.

Next-Generation Sequencing and High-Throughput Data Analysis

NGS refers to a collection of high-throughput DNA sequencing technologies. These technologies, which brought innovation to genomic studies in the late 1990s, can obtain rapid, low-cost, high-volume DNA sequence information and perform whole genome, transcriptome, and epigenome analysis. This provides researchers with an important tool for understanding the composition and functions of microbial communities. NGS has begun to be used in forensic microbiology and post-mortem analysis (Nodari et al., 2024).

Today, NGS technologies are based on various working principles. These second-generation sequencing methods include pyrosequencing (454/Roche), reversible termination (Illumina), sequencing by ligation (ABI/SOLiD), and semiconductor sequencing (Ion Torrent). Illumina is a widely used high-throughput technology that performs high-quality and accurate sequencing. However, its short read length makes it difficult to sequence and analyze large genomes. Another disadvantage is the time-consuming library creation. In addition, Ion Torrent is a low-cost technology and performs faster sequencing. Its disadvantages over Illuma include shorter read times, higher error rates, and lower throughput. Third-generation sequencing methods are SMRT sequencing (PacBio) and Nanopore sequencing (Oxford). The long reads provided by PacBio are suitable for the identification of genome assembly and structural variations. Since there is no PCR reaction, amplification errors are minimized. It is more expensive and has a low throughput compared to Illumina. Oxford Nanopore technology stands out in terms of its portability compared to other devices. Its prominent advantages are its ability to make long reads, and rapid detection, and analysis of genetic material. It does not require PCR amplification, but it performs sequencing with low accuracy compared to Illumina. In addition, it has a low throughput and is an expensive technology (Nodari et al., 2024; Minogue et al., 2019).

Cho et al. compared the 16S rRNA analyses of postmortem specimens using Sanger and NGS. Sanger sequencing of isolated bacteria was performed with MicroSeq ID, and NGS analysis was carried out on the MiSeq Illumina platform. The study, which used 65 postmortem specimens, found that MiSeq Illumina was faster and less expensive, made it easier to identify bacteria that were difficult to culture, and was more accurate (Cho et al., 2017).

NGS enables bacterial community analysis by amplifying hypervariable regions of 16S rRNA and sequencing millions of gene fragments in a single run. However, some species or phyla may be excluded from this analysis because their 16S rRNA primers do not match those used in this study. Postmortem microbial diversity analysis is performed to estimate the time of death, determine the cause of death, or determine the microbial fingerprint of an object or person and relate it to individuals or geolocations (Yang et al., 2014).

In a study investigating the soil metagenome, samples taken from 11 different environments were analyzed with the Roche/454 platform. The results showed that 18S rRNA marker analysis can be used to filter the database. Similarly, forensic and metagenome analysis of environmental soil samples enabled DNA analysis of microflora, plants, and other living organisms. In addition, DNA information obtained by NGS technologies from bacteria left on surfaces contacted by people is used in forensic research (Yang et al., 2014).

Yu et al. conducted a study in which traditional autopsy and bacterial culture were combined with NGS. In a case where nosocomial infection was suspected, the deceased's bacterial culture and NGS analysis results revealed *Enterococcus* and *Acinetobacter baumanni*, the most common pathogens in these infections. Medical records and dissection results supported the diagnosis of nosocomial infection. Combining NGS and traditional methods could help determine the true cause of death. It was noted that NGS shortened the diagnosis time and confirmed the results obtained with the traditional method (Yu et al., 2021).

The most important factor that can affect the accuracy of NGS analyses is that environmental contamination prevents the detection of exogenous microorganisms. In this sense, meticulous sampling and processing are of great importance. To determine the true microbial footprint, proper bioinformatics protocols must be followed. In addition, forensic samples generally show low biomass, which can make it difficult to obtain sufficient genetic material for analysis. Fragmented DNA sequence information obtained from analyses performed with degraded DNA may not enable true microbial identification (Nodari et al., 2024).

Machine Learning and Forensic Microbiology

It is impossible for humans to comprehend the complex information of microorganisms at the molecular level and generate a meaningful pattern from it. Machine learning is the term used today to describe the use of artificial intelligence to make sense of complex data. High-throughput data sets generated by NGS technologies have increased the use of microbiomes in forensics by combining them with machine learning. While traditional statistical methods are used to determine the overall composition of the microbial community, machine learning models can make precise predictions and perform quantitative analyses. Nowadays, the use of machine learning models like random forest (RF), support vector machine (SVM), and AdaBoost in forensic investigations is regarded as a promising development. (Goodswen et al., 2021; Yuan et al., 2023). Figure 1 shows the application areas of machine learning.

Postmortem interval (PMI) estimation is an important task for forensic investigation evaluation. A study conducted with mice found that the dominant phyla in the oral cavity were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes when PMI was 0 h. It was observed that Proteobacteria and Firmicutes were still dominant 240 h after the death of mice. Since the changes in the oral microbiota of mice and humans would be different, linear regression models were created between PMI and relative abundance. As a result, Gamma-proteobacteria and Proteus species were shown to be the

most suitable candidates for PMI estimation (Dong et al., 2019). Liu et al. reported that postmortem microbial community analysis could be estimated (1.5 ± 0.8 h within 24-h decomposition and 14.5 ± 4.4 h within 15-day decomposition) by combining artificial neural network (ANN) and postmortem microbial data set (Liu et al., 2020).

Individual identification is also an important aspect of forensic microbiology. Identifying the microbiome in skin, hair, or touching objects is critical, particularly when there is no evidence of blood or tissue. A study found that combining the skin microbiome and Cutibacterium acnes 16S rRNA genotype with random forest machine learning resulted in 90% accurate person identification. In another study where person identification was made with skin microorganisms, a novel sequencing panel (hidSkinPlex) was created. In the study where samples were taken from three different body regions (hand, foot, and manubrium) of eight individuals, regularized multinomial logistic regression and 1-nearest-neighbor classification were used to determine the accuracy rate ranging from 52.20 to 100% (He et al., 2022).

Identification of biological traces and knowledge of their origins are important for forensic sciences. However, exposure of tissues and fluids to environmental effects makes identification difficult. At this point, identifying the microorganisms in body fluids such as skin, peripheral blood, menstrual blood, saliva, vaginal secretions, and semen may be critical (He et al., 2022). In a study conducted on the subject, skin, saliva, and vaginal fluid classification was achieved with NGS technology and taxonomy-independent deep learning networks. Body-site classification accuracy values were determined as 0.99 for skin, 0.99 for oral, and 1 for vaginal secretion (Díez López et al., 2020). Microbial biomarkers for the human body were created from mixed samples created from different body parts and soil. 635 operational taxonomic units were determined as biomarkers with Generalized Local Learning. It has been reported that Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria biomarkers are dominant in all body regions, and Firmicutes and Bacteroidetes are in high fractions in

feces and Proteobacteria in the skin (Tackmann et al., 2018).

Geographical identification is a relatively new task in forensics. In forensic investigations, evidence that will show the relation of a victim, suspect, or object to a crime scene is important. The International Metagenomics and Metadesign of Subways and Urban Biomes (MetaSUB), which was launched in 2015, is a global network of scientists and clinicians. It was developed to obtain urban microbiome information on transit systems, built environments, and hospitals. A sequence of approximately 5,000 samples was obtained using NGS technologies, and the geographic origin relationship was evaluated using bioinformatics and artificial intelligence algorithms. Researchers used machine learning techniques to determine the origin of microbiome samples. It was reported that 90% of the samples were classified correctly. This study stated that machine learning can be used in the field of biogeography, but further evidence is needed to expand the application (He et al., 2022).

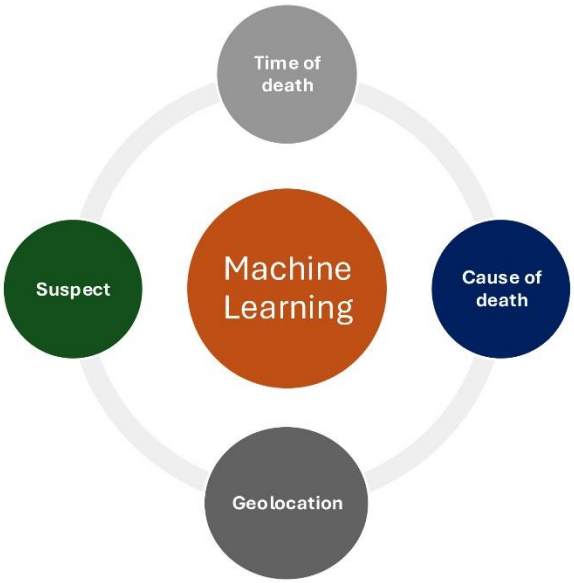

Figure 1. Application of machine learning in forensic investigations

In addition, algorithms that can be applied to cultural methods have been developed with artificial intelligence applications. 'Chromogenic media image detection' is one of these algorithms. Surveillance of cultures is an improved computational image analysis technique. Some studies have reported that it detects high sensitivity and specificity in positive cultures. The best application of the 'Colony counting with Growth versus No-growth Discrimination' algorithm may be for bulk samples. A deep convolutional neural network has been developed for culture analysis of urine samples. Easy and fast discrimination can be made with the 'Phenotypic Colony Recognition', an image analysis algorithm that distinguishes positive cultures from negative cultures (Mishra et al., 2023).

The Potential of Deep Learning

Today, AI systems are often based on machine learning. Machine learning refers to the ability of systems to learn from problem-specific training data in order to automate the process of developing analytical models and solving related tasks. Deep learning is a machine learning concept that utilizes artificial neural networks. For many applications, deep learning models outperform shallow machine learning models, and traditional data analysis approaches (Janiesch et al., 2021.)

Although forensic microbiology is defined as the identification of the microbial source of the environment or the host, in recent years, it has also included the determination of the spread of pathogens among individuals and the assessment of the damage caused. In this regard, Yao and Zhang discussed the link between human papillomavirus (HPV) and cervical cancer, as well as machine learning, deep learning, and medical examination data used to detect HPV infection (Yao & Zhang, 2023).

Recent research has demonstrated that convolutional neural network models can distinguish between bacteria and algae based on microbiome images. This model focuses on the segmentation, clustering, classification, and counting of microorganisms. Tuberculosis was identified with 78.4% accuracy in microscopic smear images. Deep learning, which can be a strategy in the diagnosis of deaths by drowning, is the identification of diatoms and algae with image analysis (Yuan et al., 2023).

The accurate identification of human epithelial material of salivary, skin, and vaginal origin is critical for crime reconstruction. However, the overlapping cell type composition of these three origins makes differentiation and identification difficult when the previously suggested biomarkers are applied. In the study conducted by taking advantage of the microbiota differences in these regions, 16S rRNA sequences obtained by NGS in HMP were distinguished using deep learning networks. The study showed that with the microbiome approach, accurate tissue-type classification of three epithelial materials could be made that can be associated with future forensic investigations (Díez López et al., 2019).

Future Aspects

Although many technologies and methods have been developed in forensic microbiology over the years, it can still be considered a developing field. In the future, the increase in genomic data sets with NGS technologies, as well as the emergence of higher accuracy models with artificial intelligence applications, will drive progress in this area. However, discussing some of the current issues surrounding artificial intelligence applications is worthwhile. There are limitations in judicial processes when evaluating reports generated by artificial intelligence. Examples of these limitations include the unknown nature of these algorithms, known as the 'black box', as well as the possibility that data entry and evaluation will be difficult for the judiciary to understand. The unpredictable development of machine learning mechanisms and the risks of error or cyber-attacks should not be ignored.

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THE ROLE OF MECHANISM-BASED TOXICOLOGICAL ASSESSMENTS IN SUDDEN DEATHS

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Chapter 4

The Role of Mechanism-Based Toxicological Assessments in Sudden Deaths

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Introduction

In forensic medicine, deaths are divided into natural and unnatural. Sudden deaths are defined as deaths occurring within 24 hours of symptom onset by the World Health Organization (WHO). Sudden death occurs when it occurs unexpectedly and suddenly due to natural causes. Traumatic events cause unnatural deaths. The origin of this type of death may be an accident, homicide or suicide (Lee et al., 2022). The occurrence of sudden death in individuals without known medical conditions, or the sudden death of a person without an apparent cause, is frequently perceived as unexpected by family members. Likewise, when an individual with a diagnosed illness succumbs without exhibiting clinical manifestations typically associated with a fatal outcome, relatives often classify such events as unexpected deaths (Coll et al., 2022).

Sudden, unexpected death can occur as an outcome of a disease in any organ system. Sudden natural death can be described as a rapid end of life resulting from an illness. This type of death is considered unexpected if the deceased did not previously have symptoms of such a disease or if such symptoms were not known to the deceased's friends, relatives or physician (Senel & Mızrak, 2018). Sudden, unexpected deaths may be caused by acute-serious complications of a systemic disease, acute or late complications of a previous trauma, acute or late complications of a forgotten or neglected trauma or intoxications (Gülmen & Meral, 2011; Sessa et al., 2021). Most deaths can be documented sufficiently to explain the cause of death by a physician who treated the patient while he or she was alive or who could be presumed to have realized some aspects of the medical history. This is the best result that can

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normally be expected. However, it is not sufficient for the accuracy of the statistics because, in the majority of all deaths, an autopsy is not possible, even with public consent. In most cases, the person appears to be in good health and can perform his or her daily activities. In cases where the death is sudden and unexpected, if there is no cause to explain the death, a postmortem investigation of an autopsy is necessary to explain how the death occurred (Payne-James & Jones, 2019).

Forensic toxicology can provide valuable data in cases where death is not directly attributable to intoxication. Examples include the presence of alcohol in traffic accident victims, the presence of psychoactive substances in certain deaths related to violence, and the presence of alcohol, narcotic drugs, and hallucinogens. Conversely, negative toxicological findings in some cases may also serve to refute allegations. Similarly, in certain patients who are required to take their medication regularly, such as those with epilepsy, the absence of the requisite concentration of medication in the blood may strengthen the hypothesis that the individual succumbed to a seizure (Poklis, 1997).

Sudden cardiac death (SCD) is the predominant cause of mortality worldwide. Advanced methodologies for the investigation of potential sudden deaths associated with fatal arrhythmia are crucial for accurately determining the cause of sudden death and preventing mortality in other family members, particularly those with suspected hereditary syndromes. While forensic pathologists currently bear the primary responsibility for ascertaining the precise cause of sudden death, optimal outcomes can be achieved through collaborative interdisciplinary efforts (Campuzano et al., 2014; Kauferstein et al., 2013). Sudden arrhythmic death syndrome is hypothesized to be the underlying cause of autopsy-negative SCDs frequently encountered in forensic medicine practices and processes. In such cases, a critical inquiry pertains to the necessity of genetic analysis in all instances where toxicological investigations yield positive results for psychotropic drugs and histological and pathological examinations, including autopsy, reveal indications of structural heart disease. At present, only a limited number of academic institutions

conduct post-mortem genetic analyses (Michaud et al., 2011).

This chapter elucidates the significance of mechanism-based toxicological interpretations in differentiating sudden deaths. It explicates toxicological mechanisms that serve as direct primary causes of death, as well as those that indirectly influence mortality and augment the potential of its primary cause. Furthermore, it presents methodologies established for the interpretation and prevention of sudden deaths from a forensic toxicological perspective.

1. Toxicology and Death Investigation

The primary aim of death investigations is to provide justice to the public health system. A coroner death investigation is initiated by judicial authorities when hospital staff or law enforcement notifies the medical examiner's or coroner's office, depending on the status and location of the death (Cusack, 2023; Randall et al., 2003). The investigator involved in the forensic process obtained information about the medical history of the dead, including the circumstances in which the deceased was found for deaths outside the medical setting and an investigation of the place or scene of death was provided. Initial findings suggestive of overdose or exposure to toxic substances may be obtained from the scene of death. These may include recently witnessed symptoms, signs of illicit or prescription psychoactive substances, foamy fluid from the mouth or nose, or macroscopic evidence of chronic or acute injection sinuses ("track marks"). Toxicological analyses are an essential part of forensic pathology. Where it is suspected that drug use contributed to or caused death, autopsy not only allows samples to be taken for examination but also, more importantly, allows the coroner to exclude other possible causes of death. A more accurate toxicological interpretation of sudden death depends on additional information obtained from autopsies. The natural processes of the body sometimes shape the effects of drugs. Individuals with severe hepatic or renal impairment may exhibit alterations in drug metabolism and elimination rates. Such changes can potentially affect therapeutic concentrations or modify tolerance levels. Furthermore, postmortem toxicology screenings that yield

unexpected negative results for specific substances can provide valuable insights into the influence of natural diseases or injuries during autopsy examinations (Kastenbaum et al., 2019).

In cases of prolonged hospitalization preceding death, the utilization of validated analytical techniques for toxicological examination and assessment of underlying medical conditions demonstrated that autopsies provided minimal additional insight. Drug concentrations measured in samples obtained during postmortem examinations cannot yield reliable information regarding drug toxicity. In exceptional circumstances, fluid samples collected antemortem may prove highly valuable for determining the contribution of drugs or toxic substances to the cause and mechanism of death (Rohrig, 2019c). In cases where drug intoxication is suspected as the cause of mortality, post-mortem examinations frequently fail to yield conclusive evidence that directly correlates the fatality to substance use (Dolinak, 2013). Nevertheless, the specific internal and external findings may indicate a history of psychoactive substance use and/or the feasibility of poisoning. (Rohrig, 2019c).

External indicators, such as cicatrices or linear puncture marks along the superficial veins, frequently indicate a history of intravenous drug administration. Subcutaneous injections may result in localized ulceration, edema, and circular scarring of the epidermis. Furthermore, inhalation of illicit substances can lead to perforation of the nasal septum, erosion of the dental enamel, and crystalline deposits in the nasal cavity. In cases where substance abuse may have contributed to mortality, non-specific external manifestations may include frothy fluid in the oral and nasal cavities as well as widespread evidence of infection (petechial hemorrhage). Prolonged unconsciousness may be indicated by skin blisters or tissue death in areas with poor perfusion. In suspected fatal drug overdoses, internal examination may reveal cerebral swelling, frothy secretions in the respiratory tract, fluid accumulation in the lungs, and an enlarged urinary bladder. The ingestion of medications can be evidenced by examining the contents of the oesophagus and stomach for pills, capsules, residues, or fillers. The

gastrointestinal system is the most common site for detecting drug packages in cases of body packing or concealment. Microscopic analysis can identify foreign substances used in drug compositions or as fillers, which typically trigger an immune response. In cases of suspected drug poisoning with extended survival or unconsciousness, autopsy findings may reflect the consequences of this period. These may include complications, such as pneumonia, liver cell death, early-stage heart attack, and muscle tissue breakdown. However, these observations are not specific and generally indicate damage to multiple organs caused by insufficient oxygen supply rather than the direct harmful effects of the substance in question. Some drugs may produce more distinctive patterns of findings during postmortem examinations. However, the lack of such findings during autopsy does not rule out the possibility of toxicity. There is no single toxicology screening that can identify all drugs or poisons, and many coroner's offices face financial constraints (Kastenbaum et al., 2019; Rohrig, 2019a). Consequently, choosing the appropriate toxicological test requires careful consideration of the evidence found at the crime scene and during the autopsy, as well as knowledge of the available analyses at the toxicology laboratory used by the death investigator. This process is essential for determining the substances that should be examined.

2. Conventional Specimens in Postmortem Toxicology

Blood: Autopsy blood samples are generally considered the most reliable method for assessing a drug's potential toxicity and its contribution to death. Nevertheless, the interpretation of substance levels is complicated by the static condition of the blood post-mortem. Analysis of postmortem blood can be affected by the movement of drugs along concentration gradients, which may vary based on the properties of the substance. This diffusion process can potentially modify the measured blood concentrations of these compounds during analytical procedures (Pounder & Jones, 1990; Rohrig, 2019b). Drugs or chemicals characterized by high distribution volumes, lipophilic properties, and basic nature are more prone to post-mortem redistribution. Furthermore, pharmaceuticals can migrate into the

bloodstream or concentrate in nearby organs from the digestive system, surrounding fat deposits, or muscle tissues. (Rohrig, 2019b). To address the issue of postmortem redistribution, samples are routinely collected from both central and peripheral locations during autopsies, allowing for drug concentration comparisons when needed. In postmortem toxicological analyses, peripheral blood is considered the sample of choice due to its relative isolation from internal organs. The femoral veins are typically the preferred sites for collecting peripheral blood, as their intravascular valves often prevent the downward flow of blood from the abdominal area. When femoral blood is unavailable, the subclavian veins serve as acceptable alternative sources for peripheral blood collection (Rohrig, 2019b).

Vitreous humour: The vitreous fluid is relatively shielded from microbes and typically exhibits low enzymatic activity. As a result, it is particularly valuable for analyzing electrolytes and determining ethanol levels. Additionally, this fluid can be beneficial in identifying opioids, such as 6-monoacetylmorphine (Dolinak, 2013). The vitreous fluid's relative isolation causes a delay in the distribution of substances into it, often lagging behind the drug's absorption phase in the bloodstream. In postmortem samples, the concentrations of compounds like ethanol found in the vitreous typically reflect the blood levels present in the hours preceding death (Dolinak, 2013; Rohrig, 2019b).

Urine: Urine collected after death is relatively shielded from microbial activity, but its utility is limited to substance screening. This is because the concentration of drugs in urine has not been demonstrated to accurately correlate with blood levels (Dolinak, 2013; Pounder & Jones, 1990). The metabolism and distribution of a pharmacological agent require time before it is detectable in urine, which can result in false-negative urine tests for individuals who expire shortly after drug ingestion, prior to substantial metabolic processes occurring. Conversely, urinalysis can identify pharmacological substances that have already been metabolized by the body and are no longer detectable in the bloodstream.

Tissue: In postmortem toxicology analysis, tissue samples serve as a viable alternative when blood

samples are unavailable. The liver and skeletal muscle are the most frequently utilized tissues for this purpose. The right lobe of the liver is preferred for collection, as it minimizes diffusion from adjacent organs such as the stomach. Skeletal muscle, typically obtained from the extremities (predominantly the thigh), is generally well-preserved and anatomically isolated from the viscera. However, it is noteworthy that drug concentrations in skeletal muscle can be variable and may not accurately reflect blood drug levels at the time of death despite its relatively protected anatomical structure (Dolinak, 2013; Rohrig, 2019b).

3. Toxicology Report

Ascertaining the ethology of mortality in drug-related cases necessitates a comprehensive approach encompassing multiple disciplines. While toxicological analyses provide crucial data regarding drug concentrations, these findings must be evaluated in conjunction with the individual's medical history, postmortem examination results, and the presence of concomitant substances. It is imperative to note that an elevated drug level in isolation does not necessarily indicate that it was the primary cause of death. (Gerostamoulos, 2022; Kastenbaum et al., 2019). Individuals who chronically consume opioids develop tolerance, enabling them to maintain normal function despite elevated concentrations of these substances in their systems. Conversely, opioid-naïve individuals may experience life-threatening consequences from even minimal doses of these medications. Subjects with compromised cardiac or pulmonary function are at heightened risk of adverse effects or mortality from central nervous system (CNS) depressants compared to those with intact cardiopulmonary systems. Irrespective of the extent of cardiovascular disease in the deceased, stimulants such as cocaine and methamphetamine possess the potential to induce fatal cardiac arrhythmias at any dosage level (Dolinak, 2005). Lastly, when CNS depressants, including alcohol, from identical or different categories, are used concurrently, their effects can be cumulative. Consequently, the detection of small quantities of multiple CNS depressants may be interpreted as a combination resulting in fatal CNS suppression. Certain substances

identified in toxicological screenings might be metabolites of other drugs or the primary drug consumed. For instance, hydrocodone, an opioid medication, is metabolized into hydromorphone, which is also an opioid drug (Baselt & Cravey, 1982). Analysis of medications prescribed to a deceased individual, either through examination of drugs found at the death scene or review of prescription monitoring program records, can provide insight into which substances were likely consumed and which are metabolic byproducts. The presence of 6-monoacetylmorphine, a specific metabolite of heroin, in blood or urine samples indicates heroin usage. While morphine is also a heroin metabolite, determining whether its presence is due to heroin use or direct morphine intake may necessitate consideration of the deceased's medical history and evidence from the death scene, particularly in the absence of 6-monoacetylmorphine. Codeine, frequently detected alongside heroin, is not a known metabolite; its presence is hypothesized to result from impurities in the street heroin supply (Al-Asmari, 2020; Baselt & Cravey, 1982).

4. Determining the Cause of Death

Cause of death is "the disease or injury that initiated the chain of morbid events leading directly to death, or the circumstances of the accident or violence that caused the fatal injury" (World Health Organization, 2024). The cause of death is an opinion formed by the person issuing the death certificate (Langman & Lin, 2022). Determining the cause of death can vary in certainty. In clear-cut situations, such as a fatal heart injury from a stabbing, the medical examiner can confidently attribute death to the wound. The presence of other factors like natural ailments or alcohol in the system is unlikely to alter this conclusion. However, the interplay between natural diseases, injuries, and drug effects requires careful consideration in more complex scenarios. The person issuing the death certificate must weigh the relative impact of each factor to determine the most probable cause. Frequently, a combination of natural disease processes and injury is deemed the likely cause of death. This assessment involves evaluating how these various elements contributed to the fatal outcome rather than focusing

on a single, definitive cause (Kastenbaum et al., 2019).

A critical error forensic pathologists should avoid is concluding the cause of death based exclusively on drug concentration without examining the entire case context. Various sources provide information on toxic or fatal doses and concentrations. It is important to note that the term "lethal dose" has three distinct definitions, which are often used "indiscriminately and interchangeably" in scientific publications (Gill & Stajíc, 2012). The following are essential: 1) the lowest dose that invariably results in fatality, 2) the lowest dose that typically leads to death, and 3) the lowest known lethal dose. Accurately determining death by poisoning requires three key elements. Firstly, the post-mortem examination must not reveal any illness or physical trauma severe enough to be incompatible with survival. Secondly, toxicological findings should fall within the range typically observed in such fatalities. Thirdly, the background and circumstances should align with a fatal poisoning scenario. The initial criterion brings up the importance of conducting autopsies in suspected poisoning cases. One might question the necessity of an autopsy, given that most poisoning deaths lack visible anatomical or microscopic evidence. However, poisoning fatalities are generally diagnosed by ruling out other causes. There is no universal consensus among forensic pathologists regarding toxicological analysis of specimens from deceased individuals who have not undergone an autopsy. Some departments conduct toxicology tests on all deaths, while others limit these tests to autopsied cases or a specific subset (Schultz, 2012).

Postmortem changes cause qualitative and quantitative changes in toxicological findings (Gerostamoulos, 2022; Rohrig, 2019b). The qualitative and quantitative analysis of substances in a body after death may yield different results compared to those obtained just before death, depending on factors such as the time elapsed since death. Postmortem changes can cause certain toxic compounds to vanish, while others, like ethanol, may be generated by bacteria in the colon. To differentiate between ethanol produced after death and that consumed before death, analyses of vitreous fluid or urine can be helpful. These variations highlight the importance of considering the

postmortem interval and other factors when interpreting toxicological findings in deceased individuals. (Lin et al., 2020). Spontaneous hydrolysis of cocaine can result in the formation of benzoylecgonine. This process can be minimized by adding a preservative, such as potassium fluoride, and keeping the sample cool (Dinis-Oliveira et al., 2010). For certain substances like ethylene glycol or arsenic, simply detecting their presence may be enough to determine their role in death, regardless of the amount present. Since these substances aren't typically abused or used medically, their mere detection usually suffices. However, assessing the impact of drugs or substances of abuse on death is more challenging, particularly when the deceased had a serious underlying condition that could explain the fatality. In such instances, forensic pathologists consider both the substance concentration and the overall case context. The reliability of postmortem concentrations in determining the cause of death raises questions about how these levels correlate with concentrations before death. This is especially relevant when the deceased had a severe pre-existing illness that could account for the death (Gerostamoulos, 2022; Rohrig, 2019b).

The research on fentanyl-related fatalities investigated both postmortem and antemortem levels of fentanyl and its metabolites (Andresen et al., 2012; McIntyre et al., 2014). A study compared fentanyl concentrations in the blood of 118 deceased individuals who had been using therapeutic fentanyl with serum levels in 27 living patients utilizing fentanyl patches for medical purposes. The investigators found that postmortem blood fentanyl levels were, on average, as much as nine times higher than in vivo serum concentrations at equivalent doses. Based on these findings, they determined that direct comparisons between postmortem blood concentrations and in vivo serum concentrations of fentanyl are not feasible (Andresen et al., 2012). An additional investigation revealed that fentanyl levels in postmortem samples, including those from femoral blood, increased as the time since death lengthened (Olson et al., 2010).

In 1975, Holt and Benstead first identified postmortem redistribution by observing variations in digoxin levels across blood specimens collected from

three distinct locations (the heart, neck, and leg) (Holt & Benstead, 1975). The researchers proposed that blood samples from leg veins should be collected post-death to evaluate potential antemortem digitalis toxicity. In a 1985 study examining digoxin levels in rats at death, 12 hours later, Koren and MacLeod introduced the concept of "postmortem redistribution" (Koren & MacLeod, 1985). During one's lifespan, the constant flow of blood ensures that drug concentrations remain relatively uniform, regardless of where blood is sampled. However, after death, certain changes must occur to explain the variations in drug concentrations observed at different sampling sites. Post-mortem alterations contribute to these differences, including a reduction in blood pH and enhanced membrane permeability. As a result of these changes, drug levels in the blood become dynamic, shifting along concentration gradients. The process of post-mortem redistribution encompasses both movements within blood vessels, leading to location-specific disparities, and the transfer of drugs between tissues (Palmer, 2010). The redistribution of drugs may not be solely attributed to tissue concentration gradients, as evidenced by certain drugs with high distribution volumes, such as cannabinoids, which have not consistently shown elevated blood levels following death (Drummer, 2004). The distribution of drugs within the body can be altered when substances traverse barriers that typically maintain concentration gradients throughout an organism's lifespan. This phenomenon is particularly significant for small, non-polar molecules, which may diffuse across previously impermeable barriers (Pélissier-Alicot et al., 2003). The redistribution of drugs after death occurs due to the movement of substances from areas of high concentration to areas of low concentration following cellular membrane breakdown. This process particularly affects drugs with high lipid solubility or substantial tissue concentrations characterized by a large volume of distribution. Various factors contribute to the variability between sampling sites, including blood sample size, time since death, the type of sample (whole blood or plasma), drug distribution volume, tolerance, protein binding, and drug stability after death. When collecting peripheral blood

samples, it is crucial to avoid obtaining too large a volume, as this may include blood that has leaked from central areas. Some experts suggest tying off or compressing the proximal femoral or iliac vessels to prevent contamination of the sample with blood from central regions (Rohrig, 2019b).

Postmortem drug concentrations are influenced by various internal and external factors, making it crucial to avoid drawing incorrect conclusions about the cause of death based solely on drug levels or without considering the postmortem nature of the sample when interpreting results. The forensic pathologist or coroner is responsible for effectively communicating with the toxicology laboratory, understanding analytical limitations, and properly collecting samples. In interpreting toxicology results, the forensic pathologist/medic must consider the entire death investigation, including the deceased's medical history, death circumstances and setting, autopsy findings, and analytical toxicology outcomes. Toxicology results should only be included on the death certificate if they contribute pathologically to the death.

5. Chemicals (Drugs or Toxicants) as Secondary Cause of Sudden Deaths

The global trend of rising recreational drug use among teenagers and young adults is widely observed. Cocaine, a well-known stimulant drug that is often abused, has been associated with cardiac fatalities, similar to amphetamines. Both substances operate through comparable mechanisms, triggering the release of catecholamines such as norepinephrine, dopamine, and serotonin. This process results in coronary vasospasm and the formation of blood clots. The combined effects of these adverse reactions can induce myocardial ischemia and infarction, even in the absence of atherosclerosis. (Gagnon et al., 2022).

Cittanidi F. et al. reported on a case involving a 41-year-old individual who experienced cardiac arrest and was subsequently taken to the emergency department. Prior to the cardiac event, the patient had exhibited threatening and violent behaviour in their community. Initial tests conducted in the emergency room revealed the presence of cocaine and ethanol (1.99 g/L) in the patient's bloodstream. Despite medical interventions, the

patient succumbed to cardiac arrest 14 hours later. Following the death, a standard autopsy was conducted, with samples collected for histological, genetic, and toxicological analyses. Systemic toxicological analysis of the samples was performed in accordance with TIAFT guidelines. The results showed positive indicators for cocaine metabolites in both hair and blood samples. Furthermore, the detection of cocaethylene in the hair suggested long-term concurrent use of alcohol and cocaine. After a comprehensive examination, it was determined that the cause of death was sudden cardiac death resulting from the interaction between ethanol and cocaine, occurring in the context of Arrhythmogenic right ventricular cardiomyopathy (ARVC) (Cittadini et al., 2015). A notable aspect of this case was the underlying cause of death. The combination of cocaine and ethanol is considered more dangerous than either substance independently, as it heightens the heart's oxygen requirements. The combined effect of ethanol and cocaine significantly increases heart rate, with cocaine levels in the blood rising by up to 30%. Additionally, cocaethylene can have more severe impacts on the brain compared to using only one of these substances. Although it has similar pharmacological and psychomotor stimulant effects, cocaethylene has a longer half-life than cocaine and is believed to be more lethal. Ethylene is linked to a 40-fold higher risk of acute cardiac events and a 25-fold greater risk of sudden death (Pennings et al., 2002; Pergolizzi et al., 2022). As far as we know, there have been no published cases of sudden death attributed to cocaine's effects in individuals with ARVC. Determining the exact impact of cocaine on humans is challenging due to various factors, including administration methods, dosage variations, pre-existing risk factors, and simultaneous use or abuse of other substances that may interact, such as alcohol, caffeine, and amphetamines. Cocaine use has been linked to numerous cardiovascular conditions, including acute myocardial ischemia and infarction, arrhythmias and sudden death, myocarditis, cardiomyopathy, hypertension, aortic rupture, cerebrovascular aneurysm, accelerated atherosclerosis, and endocarditis (Pergolizzi et al., 2021). The ongoing debate in the literature regarding cocaine's cardiac effects makes it

challenging to draw definitive conclusions. However, it appears that the combination of cocaine and ethanol, resulting in cocaethylene formation, likely worsened the existing ARVC condition. The current body of research lacks sufficient information to propose a reliable hypothesis for the etiopathogenesis in this instance, particularly in distinguishing between the drug-induced effect and its synergistic impact on a purely anatomical structural disorder. Furthermore, it is crucial to acknowledge the near impossibility of quantifying the influence of individual variability, metabolic tolerance to the substance, and neuroadaptation on the observed effects (Cittadini et al., 2015).

According to estimates for World Drug Report 2024, approximately 228 million individuals worldwide between the ages of 15 and 64 have used cannabis at least once in 2022. (United Nations Office on Drugs and Crime (UNODC), 2024). Cannabis is widely used illicitly, with increasing legalization in numerous countries. It induces cardiovascular side effects, although the associated adverse reactions are generally limited. The psychological and physiological effects of cannabinoids have been demonstrated in previous studies. It elevates blood pressure and heart rate and can precipitate arrhythmias and myocardial ischemia. (Goyal et al., 2017). While cannabinoids have low acute toxicity, there is limited public knowledge about the potential cardiovascular risks associated with cannabis use. Some cases of cannabis intoxication have been reported without comprehensive histopathological examination. In postmortem toxicological analyses of cannabis intoxication incidents, THC concentrations in whole blood were found to range from 2 to 22 µg/L. However, these investigations lacked complete autopsies, histopathological examinations, and genetic and serum markers of myocardial necrosis. Despite the current perception that the absolute risk of cannabis-induced myocardial infarction is low and arrhythmias are reversible, individuals with cardiovascular disease risk factors should refrain from using cannabis. The presented cases underscore the potential cardiovascular dangers of cannabis use in seemingly healthy young people. Nevertheless, predicting an individual's response to cannabis ingestion remains challenging due to

underlying health conditions and complicating factors in certain individuals (Hartung et al., 2014). In contrast to cigarettes, which have high nicotine content, cannabis is believed to cause a more significant increase in heart rate, cardiac output, blood pressure, and venous carboxyhaemoglobin levels. These effects substantially decrease the time one can exercise before experiencing anginal symptoms. Although a few instances of cannabinoid-induced arrhythmias and myocardial infarction have been documented, none of these reports included information on blood THC levels at the time of the cardiovascular event. Certain medications, particularly antipsychotics, have a strong link to SCD. The suggested mechanisms involve the development of myocarditis and the ability to extend the QT interval, potentially leading to life-threatening arrhythmia (X.-Q. Li et al., 2021). According to the standards adopted by the American Academy of Forensic Sciences and the Society of Forensic Toxicologists, various specimens should be analyzed, including blood from the heart, blood from the femoral vein (peripheral), urine, or bile.

The reports have documented unexpected fatalities resulting from the concurrent consumption of ethanol and disulfiram, a medication used in alcohol addiction treatment. Disulfiram irreversibly blocks the enzyme aldehyde dehydrogenase, accumulating unmetabolized aldehydes that create an aversion to alcohol. The full effect of a therapeutic disulfiram dose takes 12 hours to manifest. These sudden deaths are attributed to acetaldehyde toxicity. While ethanol exhibits cardiotoxic properties, acetaldehyde is known for its hepatotoxic effects. In a 2006 study, Gürcan Altun et al. observed that simultaneous intake of disulfiram and ethanol triggered myocardial infarction. They advised administering disulfiram 24 hours after alcohol consumption in alcoholism treatment and emphasized the importance of patient education regarding this risk. Similar interactions have been noted with the antibiotic metronidazole. A post-mortem examination of a 31-year-old chronic alcohol user with minor injuries revealed the presence of acetaldehyde, ethanol, and metronidazole in toxicological tests. The cause of death was determined to be cardiac arrest resulting from acetaldehyde toxicity

induced by the interaction between metronidazole and ethanol (Altun et al., 2006). Another antibiotic, cefuroxime, was also linked to a comparable incident of unexpected fatality when combined with alcohol (Dong et al., 2013).

Asthma, a long-term inflammatory condition, causes damage to the respiratory system. Substance misuse is recognized as a factor that intensifies asthma activity and increases its mortality and morbidity rates. Research has documented cases of asthma patients whose deaths were associated with the presence of illegal drugs in their systems. While investigations have established a connection between drug abuse and asthma-related fatalities, the exact mechanisms by which opiates worsen asthma remain uncertain. Multiple factors likely influence this process. For instance, opiates in the bloodstream promote histamine release, which activates the parasympathetic nervous system, leading to constriction of pulmonary smooth muscle.

Additionally, heroin has been shown to cause mast cell degranulation and trigger inflammatory mediators. Moreover, opioids induce pulmonary oedema and suppress the respiratory-stabilizing functions of the central nervous system (Serinelli et al., 2020). In a study conducted by Hlavaty et al., the impact of opiates on unexpected asthma-related fatalities was investigated among 94 asthma patients. Autopsy findings, based on microscopic examination, attributed 68 cases to asthma. Toxicological screening detected opioids in 20 out of the 68 patients. Nevertheless, opiates were determined to be a direct contributing factor to death in only two cases (Hlavaty et al., 2015). Numerous antipsychotic and antidepressant medications are known to prolong QT interval and/or induce TdP arrhythmias, thereby increasing the likelihood of ventricular arrhythmias and subsequent SCD. These pharmacological agents may also exacerbate the risk of ventricular arrhythmias and sudden cardiac death by eliciting the Brugada syndrome phenotype. Antipsychotics can present cardiac risks even at low doses, whereas antidepressants typically manifest such risks at high doses or when administered concomitantly with other medications (Ray et al., 2009; Sicouri & Antzelevitch, 2008).

Unintentional drowning ranks among the leading causes of injury-related fatalities worldwide. In the field of forensic medicine, drowning is defined as death resulting from fluid inhalation or submersion in the airways. Examining bodies recovered from water presents significant challenges in forensic investigations. Determining the accurate cause and manner of death necessitates collaborative efforts between law enforcement, forensic physicians, and toxicologists. This process requires comprehensive knowledge of the incident circumstances, victim characteristics, medical history, and postmortem findings. Toxicological analysis can reveal the presence of alcohol and drugs, offering insights into the deceased's medical background, substance use history, and potential impairment at the time of death. Epidemiological studies have identified alcohol as the most significant factor contributing to fatal drownings, with hypnosedatives and illicit substances, particularly amphetamines, also posing risks. Research indicates that microbial contamination and fermentation can lead to postmortem alcohol production in submerged bodies, a phenomenon exacerbated by higher water temperatures and extended submersion periods. The accuracy of blood alcohol concentration measurements can be verified through various methods, such as comparing blood, urine, and vitreous alcohol concentration ratios with established reference values or analyzing for non-oxidative alcohol metabolites, which suggest antemortem alcohol consumption. While postmortem changes in alcohol levels are well-documented, less is known about the long-term stability of drugs in aquatic environments (Ojanperä & Kriikku, 2024).

6. Toxicological Aspects of Sudden Unexpected Death in Epilepsy (SUDEP)

Sudden unexpected death in epilepsy (SUDEP) is defined as "sudden, unexpected, witnessed or not witnessed, non-traumatic and non-drowning death in epilepsy patients, with or without evidence of seizures, and without a toxicologic or anatomic cause for death on postmortem examination, except documented status epilepticus" (Shankar et al., 2017). In suspected cases of Sudden Unexpected Death in Epilepsy (SUDEP), it should be

required to conduct a comprehensive autopsy, encompassing both external and internal examinations, along with a toxicological analysis of antiepileptic drug (AED) concentrations. Regrettably, many SUDEP incidents occur without witnesses, leaving no information about the victim's final moments or potential indications of seizures prior to death. Furthermore, the majority of SUDEP cases reveal that either none or all of the AEDs were at subtherapeutic levels during postmortem examination (Thom et al., 2018). Establishing epilepsy as the cause of death is often difficult due to limited evidence. Current published case series have not revealed definitive pathological features or biomarkers for SUDEP diagnosis (Barranco et al., 2020). The most frequently examined mechanisms are neural connections among the heart, brain, and respiratory systems. Seizure activity originating in or spreading to the central autonomic network can interfere with its functional connectivity. This disruption occurs by either suppressing or stimulating the autonomic regions, leading to various autonomic symptoms. These symptoms may include dysfunction of the cardiovascular and respiratory systems as well as damage to the brainstem (Costagliola et al., 2021; Manolis et al., 2019). Substantial research indicates that genetic components may contribute to the occurrence of SUDEP. Genes linked to cardiac conditions, such as long QT syndrome, bradycardia, and sudden cardiac death, can trigger both epilepsy and arrhythmias or increase the risk of seizure-induced arrhythmias. These genetic factors have been linked to SUDEP (Yan et al., 2023). Additionally, certain AEDs can exacerbate patients' conditions and trigger other health issues, including heart attacks, irregular heartbeats, and even fatal cardiovascular events such as heart-related deaths or SUDEP (Olesen et al., 2011). These investigations offer potential insights into the mechanisms behind SUDEP. Nevertheless, the root cause and exact pathological processes leading to SUDEP remain elusive. In a study by Yan et al., postmortem blood analysis of 388 SUDEP cases revealed that 218 (56.2%) had subtherapeutic or undetectable levels of antiepileptic drugs (Yan et al., 2023). Decreased concentrations of AEDs in postmortem blood samples have been proposed as a significant indicator of SUDEP. These reduced AED levels may suggest

inadequate dosage or non-adherence to prescribed medication regimens prior to the individual's death (Lund & Gormsen, 2009). Nevertheless, certain research indicated that identifying subtherapeutic AED concentrations during postmortem examinations may not be particularly useful in establishing the cause of death. This is due to uncertainties regarding the relationship between blood levels measured after death, serum levels before death, and the established therapeutic range (Lathers et al., 2011). A recent investigation examining 13 cases of SUDEP and 18 non-SUDEP cases revealed no significant differences in antiepileptic drug utilization between the groups based on postmortem toxicological analysis. This recent investigation analyzed the patterns of antiepileptic drug use in both categories of cases (Zhang et al., 2022). While the association between SUDEP and AEDs at subtherapeutic or non-subtherapeutic levels remains a subject of ongoing debate, significant attention has been directed toward the concomitant use of antipsychotic medications, which are hypothesized to contribute to the underlying mechanism of SUDEP. Individuals with epilepsy frequently present with psychiatric comorbidities, potentially resulting in a higher prevalence of antipsychotic drug prescriptions in this population compared to the general populace (Lu et al., 2021). The use of antipsychotic drugs can lead to heart-related complications. These adverse effects range from changes in cardiovascular parameters, such as heart rate and blood pressure, to more critical and potentially life-threatening conditions. Among the most serious consequences are prolongation of the QTc interval, congestive heart failure, and, in some instances, unexpected sudden cardiac death (L. Li et al., 2022; X.-Q. Li et al., 2021). Consequently, evaluating the safety and effectiveness of antipsychotic drugs in epileptic individuals is crucial, as well as examining their potential role in SUDEP.

One of the most critical challenges confronting forensic toxicologists, pathologists, and physicians is determining the cause and mechanism of sudden, unexpected death. Given the legal requirement to establish these factors based on medical and scientific evidence, a comprehensive evaluation is imperative. When analyzing drug-

related sudden deaths, several factors must be considered beyond merely quantifying the drug's blood concentration. These include a thorough autopsy, an examination of how histopathological findings correlate with the drug, the individual's demographic, clinical, and potentially genetic profile, the route of drug administration, dosage and frequency, tolerance levels, and possible interactions between multiple drugs. This multifaceted approach is essential for accurately interpreting the circumstances surrounding such fatalities.

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PERFORMANCE-ENHANCING DRUG TESTING

Berfin Sude ERTAS, Nebile DAGLIOGLU

Chapter 5

Performance-Enhancing Drug Testing

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Introduction

Forensic toxicology applies toxicological principles to medico-legal contexts. This field is broadly categorized into three primary sub-disciplines: human performance and behavioral toxicology, postmortem toxicology, and workplace testing. Postmortem toxicology involves investigating the toxicological factors contributing to an individual's death. Workplace testing aims to monitor employees' substance use upon entry and randomly, thereby ensuring safety within the workplace (Akgür & Daglioglu, 2018; Wyman, 2012). Human performance and behavioral toxicology focuses on detecting both legal and illegal substances in biological samples and interpreting their effects on individuals' behavior and performance (Akgür & Daglioglu, 2018). Additionally, this sub-discipline encompasses doping analyses, wherein the World Anti-Doping Agency (WADA) oversees the monitoring of athletes' use of performance-enhancing drugs (PEDs) (Wyman, 2012).

Over the past 30 to 40 years, the use of performance-enhancing drugs (PEDs) by athletes has gained significant recognition. Beyond professional sports, PEDs are also extensively used by non-athletes for aesthetic enhancement, muscle strengthening, and body shaping. This trend has raised public health and safety concerns in many countries (Crouch & Shelby, 2020; Odoardi et al., 2023; Piatkowski et al., 2022; Shimko et al., 2021; Van de Ven et al., 2018). To mitigate PED use, some sponsoring organizations have established testing protocols (Crouch & Shelby, 2020). The World Anti-Doping Agency (WADA) is a leading international organization specializing in anti-doping analysis.

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Established in 1999, WADA's primary objective is to eliminate performance-enhancing substances in sports. Its core functions include developing and coordinating anti-doping rules and policies across all sports and countries (WADA, 2024). Despite WADA's comprehensive list of prohibited substances, the availability of PEDs remains a concern, as these drugs can be obtained from pharmacies, supplement shops, and illicit black markets (Vlad et al., 2018).

Common biological samples used in routine doping analyses include urine (Andersen & Linnet, 2014), blood (de la Torre et al., 2021), hair (Odoardi et al., 2023), serum and plasma (Protti et al., 2019), nails (Gheddar et al., 2024), and saliva (Feng et al., 2023). Urine samples are considered optimal for routine doping controls because substance concentrations are typically higher than in blood (Rivier, 2018). Routine analyses commence with a screening phase, followed by a confirmation phase. The screening phase must be rapid, selective, and sufficiently sensitive to minimize false negatives and false positives (Protti et al., 2019). If a screening result is positive, the identified compounds and metabolites undergo targeted confirmation, and the results are subsequently verified. Given the wide range of prohibited substances, WADA-accredited laboratories prioritize using mass spectrometry (MS)-based methodologies and employ multiple analytical techniques concurrently. Notable examples include liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS), both of which offer high selectivity and sensitivity (Protti et al., 2019; WADA, 2016a). These methodologies underscore the importance of both qualitative and quantitative analyses of PEDs within forensic toxicology.

The forensic toxicological analysis process comprises three distinct stages: pre-analytical, analytical, and post-analytical. This chapter will elucidate each of these stages concerning PEDs in biological samples, addressing the challenges and constraints inherent in their interpretation. Additionally, it will present case reports, focusing particularly on the interpretation of results during the post-analytical stage. Finally, the chapter will offer

recommendations on the impact of PEDs on public health and safety.

1. Pre-Analytical Phase

When professional athletes use performance-enhancing drugs (PEDs) and test positive for doping, they commit a serious sports offense and face numerous consequences. These may include the revocation of championship titles, disqualification from future competitions, damage to their reputations, and compromised health. PED use is considered a deliberate action (Kozhuharov et al., 2022).

Accurate interpretation of analytical results requires the involvement of forensic toxicologists at all stages of the process, ensuring the proper selection, collection, and transfer of samples according to the specifics of each case. Sample selection is based on the case history and is determined by the information sought and the target analyte. For instance, urine samples are used to detect substance use, while blood samples are chosen to determine whether an individual is under the influence of a substance (Lappas & Lappas, 2021).

The time elapsed since substance intake should guide the choice of sample. Blood is selected for short-term detection, urine for up to a week, and hair for long-term substance detection. Unlike urine samples, blood reflects recent substance intake, making it preferable for detecting recent use but unsuitable for cases where intake occurred earlier. Additionally, blood collection is invasive. Hair and follicle analysis can detect chronic use and substances consumed long ago; however, they do not provide information about recent intake, and results may vary based on an individual's ethnicity (Akgür & Daglioglu, 2018). Urine is often preferred for its ease of collection, non-invasiveness, and the ability to obtain larger quantities (Protti et al., 2019). Substances can be detected in urine for longer periods than blood (Aydoğdu et al., 2021). However, urine samples must be collected under supervision to prevent adulteration, which may exceed privacy boundaries (Jacques et al., 2022).

Individuals have attempted to adulterate drug test results to conceal the use of illicit or prohibited substances (Peat, 2013). In forensic toxicological

analysis, collected samples are typically placed in evidence bags and promptly transported to the laboratory along with chain of custody forms. No biological sample is analyzed without a proper chain of custody. Samples for immediate analysis are stored at +4°C, while those for long-term storage are kept at -20°C or -80°C. However, keratinized samples such as hair and nails, which are highly stable at room temperature, can be stored under ambient conditions. When blood samples are collected, preservatives must be added to prevent degradation. Commonly used preservatives include sodium fluoride-potassium oxalate, potassium fluoride, or EDTA (Dinis-Oliveira et al., 2010).

During pre-analytical analysis, integrity tests on the collected biological samples are essential. Unlike other substance tests, doping samples are collected directly under supervision to prevent adulteration. The process begins by verifying the individual's identity to ensure the correct person is sampled and proceeds with attention to other critical factors. Urine samples can be diluted with toilet or tap water; therefore, adding colored dye to bathroom water serves as a preventive measure. Synthetic urine can be obtained online and presented as a real sample, necessitating body searches and ensuring that urine samples are at least 40 mL in volume. Following collection, the sample's temperature is measured, and it is examined for unusual odor or color. The samples are then placed in appropriate storage containers and sent to the laboratory while maintaining the chain of custody (Akgür & Daglioglu, 2018).

Guidelines have been published to standardize the collection of urine specimens and prevent sample adulteration. One such guideline, issued by the Department of Transportation (DOT), defines diluted urine samples as those with a specific gravity of less than 1.003 and creatinine levels below 20 mg/dL (Peat, 2013). Urine samples are collected as A and B samples. If the A sample is positive, the individual may request analysis of the B sample. For doping analyses, a minimum of 70 mL of urine must be collected. When blood samples are required, a volume between 3 and 10 mL is sufficient (Rivier, 2018).

When collecting hair samples, it is essential to document their characteristics to ensure accurate

interpretation of results. This documentation should include hair color, length, and any treatments applied (such as straightening, bleaching, dyeing, or perming) (Dinis-Oliveira et al., 2010). Hair samples should be taken from the posterior vertex region of the scalp, with hair thickness approximately the diameter of a pencil (at least 200 mg). It is crucial to ensure that cutting tools are sterile and that the cutting process is performed as close to the scalp as possible. If hair samples cannot be obtained from the scalp, body hair from other areas, such as the chest, pubic region, or underarms, may be collected instead (Curtis & Greenberg, 2008; Vearrier et al., 2010).

Environmental contamination is a significant consideration in hair sample analysis. Various substances can adhere to individuals' hair through proximity to those using illegal substances or by touching contaminated surfaces. This can lead to false positive results if not properly addressed (Cooper, 2015). To prevent this, a proper washing procedure should be performed during the preparatory phase, helping to differentiate between substances used by the individual and those acquired through environmental contamination (Curtis & Greenberg, 2008).

Several variables can affect the presence of substances in hair analysis. A lack of understanding of these variables or the degradation of substances over time may result in misinterpretation. Hair morphology and physicochemical properties influence the stability of substances in hair (Cooper, 2015). When selecting a biological sample based on the target analyte, it is important to consider the analyte's detectability within that sample. For example, substances with acidic structures bind weakly to melanin in the hair matrix, making their detection challenging. Examples include the acidic metabolites of tetrahydrocannabinol (THC) and most diuretics (Kintz, 2024; Scholz et al., 2022). Single doses of diuretics are unlikely to be detected in hair, as they may not effectively transfer into the hair matrix (Gheddar et al., 2019; Kintz, 2024).

In the pre-analytical stage, samples are extracted and prepared for analysis. It is important to use the most appropriate extraction methods based on the biological sample and target analyte. Extraction methods are generally

divided into three categories: solid-phase, liquid-liquid, and direct injection. In the liquid-liquid method, biological samples such as urine or blood are extracted using a buffer with an appropriate pH, followed by the use of solvents (e.g., alcohols, ethyl acetate) to separate the target analyte from the matrix. Solid-phase extraction (SPE) utilizes solid sorbents, allowing for the analysis of a broader range of polarities and using less solvent compared to liquid-liquid extraction. The direct injection method involves injecting substances directly into devices such as gas chromatography (GC), bypassing the extraction phase altogether (Drummer, 2018). When samples are to be analyzed, the most appropriate extraction method should be selected, and recovery calculations must be performed. Once extraction is complete, the pre-analytical stage concludes.

2. Analytical Phase

In conducting a toxicological analysis, the method employed must be validated. If the method has not been validated, it is not possible to assert the reliability of the results obtained. Accurate and precise target analyte detection can only be achieved through a validated method. Limit of detection (LOD) and limit of quantitation (LOQ) must be established. Quality control samples containing known analytes should be analyzed alongside actual samples in every analysis. Internal standards with similar structures to the analytes being examined should be used during analyses to ensure in-device control of the samples. Additionally, the internal standards added before extraction correct for low recovery rates (Drummer, 2018).

Expert reports provided in the context of forensic toxicological studies are legally valid documents. Consequently, the preparation of an inaccurate report will result in the imposition of inappropriate legal sanctions and the loss of individuals' rights. The reliability of expert reports is ensured through the quality of analytical methods established via method validation, thereby preventing unjust legal outcomes. Experts may choose a method published in the literature or use a method they have developed themselves. In either case, validation studies must be conducted during the method selection process.

Following the arrival of samples to the laboratory for analysis, preliminary

screening tests such as immunoassays or screening tests using GC/MS or LC/MS are employed. The method must be validated for the analyzed biological samples. For example, specific preliminary screening tests designed for blood samples should be utilized and validated for that matrix. If the results of the preliminary screening tests are included in the report, it should be noted that the results are only verified if another confirmation method has been employed. In cases where the target analytes are analyzed using a preliminary screening test or another method, a different method based on a distinct chemical principle should be applied whenever possible. This approach ensures the verification of results. Validation tests are expected to be more specific than the initial test. At this stage, the application of mass spectrometry, considered the gold standard, is recommended (Cooper et al., 2010). Therefore, samples are subjected to a preliminary screening test. A second analysis is conducted using a confirmation test if the result is positive. If this result is also positive, the toxicologist reports it as positive. If the confirmation test is negative, it is reported as negative. If the preliminary screening test result is negative, no confirmation test is performed, and the results are reported as negative (Figure 1).

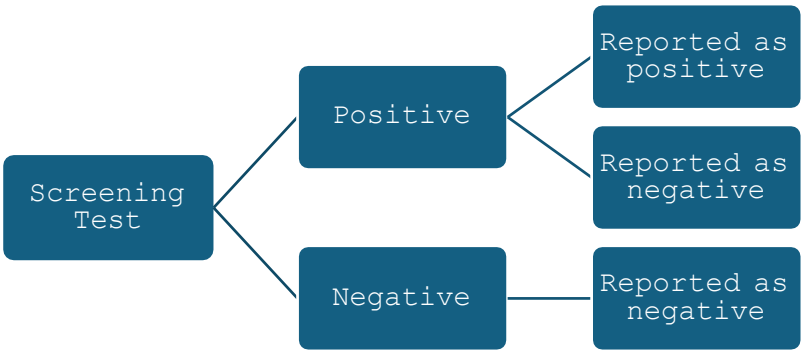

Figure 1. A summary of the analysis and reporting processes is given.

During the analytical phase, there is a possibility of misreporting due to the influence of certain factors. Such occurrences may occur in the preliminary screening tests and the confirmation phase. It is established that substances present in urine exhibit cross-reactivity when immunochemical methods are employed. Preliminary screening tests are based on the

antigen-antibody relationship. False negativity and positivity may be observed. Cross-reaction can be expressed as a situation in which the method gives false positives in substances structurally different from the target analyte but with similar antigen determinants. False negativity occurs when the chemical environment prevents the binding of the antigen of the analyte with the antibody of the pre-screening test. For example, amphetamine prescreening tests cross-react with drugs such as phenethylamine, ephedrine, proguanil, and labetalol. In addition, the biological sample may contain chemicals that directly interact with, bind to, or destroy the analyte (Ordu & Akfırat, 2018; Reisfield et al., 2009). Additionally, depending on the screening test used, both morphine and morphine-3-glucuronide can be detected, while other tests may only identify the parent compound. As a result, not all substances present in the urine may be detected. This is where adulteration with urine samples can have an effect; it can prevent the interaction between the antigen (analyte) in the matrix and the antibodies in the test, leading to false negatives. Furthermore, preliminary screening tests are designed explicitly for specific substances. Consequently, antibodies may have a limited range and can only affect a predefined number of compounds. When new synthetic substances, such as novel psychoactive substances, are continuously introduced into the market, detection of these compounds may not be possible (Ordu & Akfırat, 2018). Forensic toxicologists must be aware of these issues, carefully interpret the results, and validate the findings of preliminary screening tests using chromatographic methods.

Forensic toxicologists must be meticulous and attentive at this stage, ensuring that each phase is carefully controlled. Scholz et al. (2022) demonstrated in their study that the selection of the ion source in LC-MS can lead to false positives. The Atmospheric Pressure Chemical Ionization (APCI) ion source operates at 450°C, creating a high-temperature environment. They found that Δ^9-THCA-A degrades when the APCI ion source is used, resulting in decarboxylation and conversion to Δ^9-THC (Scholz et al., 2022).

The structural similarity between Δ^8-THC and Δ^9-THC has led researchers to suspect interference, as the structural differences between the two are minimal. Δ^8-THC differentiates from Δ^9-THC by the position of a single double bond. In a study conducted by Hart vd. (2024), the effect of Δ^8-THC on Δ^9-THC was investigated. The spike experiments revealed that the peak of Δ^8-THC could interfere with the peak of Δ^9-THC. Consequently, samples were sent to 23 different laboratories for control purposes. Of these, 20 laboratories conducted accurate analyses, while three laboratories produced incorrect results. In one of these laboratories, Δ^8-THC was present at a concentration of 20 ng/mL, yet the presence of Δ^9-THC could not be confirmed. To obtain correct results, it was necessary to modify the gradient program. The existing gradient program in the laboratory's analytical device was too short, so it was restructured according to the retention time of the Δ^9-THC peak. In the other two laboratories, before submitting the samples to the GC-MS device, one used Propyl iodide (C_3H_7I), and the other used Methyl iodide (CH_3I). After detecting the errors, all three laboratories restructured their methods and successfully achieved the separation of the substances (Hart et al., 2024).

3. Post-Analytical Phase

Toxicologists use the results of the analyses conducted in the post-analytical phase to prepare a scientific expert report. All stages of the pre-analytical, analytical, and post-analytical processes are factors that affect accurate reporting. An error at any of these stages may result in deficiencies in the report. This section presents case studies published in the literature related to the post-analytical analysis phase. The following case reports include false positives in athletes due to consuming meat, supplements, pharmaceuticals, and close contact between romantic partners.

Case 1: Kintz and Gheddar (2024) stated that when faced with such a situation, the most crucial approach for an athlete to defend themselves and prove their innocence is to conduct a hair test. In this study, Ostarine, one of the most popular selective androgen receptor modulators (SARMs), was detected in athletes who

shared the same bandage. Unlike other cases generally reported in the literature, this study presents a contamination incident involving athletes who shared the same bandage, with one athlete using Ostarine while the other did not. In this context, a new form of contamination has emerged. A male athlete underwent two doping tests over two weeks, both of which detected low concentrations of Ostarine in his urine samples. Since he firmly denied using the substance, contamination was considered, and the supplements he used were analyzed. However, the results were negative, indicating no contamination from dietary supplements. Subsequently, hair and nail samples were collected and analyzed, yielding negative results again. It was later discovered that this athlete had a fellow athlete using Ostarine, and all of the keratinized samples from that athlete tested positive. It was determined that the athlete using the substance had been using the same bandage as another athlete who did not use it during this period. Consequently, it was suspected that the substance transferred through sweating due to their shared use of the same product. Additionally, 12 samples weighing 1 gram each were collected from the bandage and analyzed, revealing a positive result for Ostarine (3-142 pg/g). It was also noted that the athlete exposed to contamination had used a cream during this time and using a bandage while applying the cream could lead to transdermal absorption of the substance. All obtained results were evaluated together, and subsequently, the United States Anti-Doping Agency (USADA) concluded that the substance transfer was due to the bandage use and did not penalize the athlete. WADA received the case but did not appeal the decision (Kintz & Gheddar, 2024a).

Case 2: A critical note of caution during interpretation is the possibility of a positive result in hair analysis despite the individual not using the substance. This can occur if the individual is exposed to an environment where the substance is smoked, leading to passive inhalation or through direct contact with the substance, such as handling it and inadvertently transferring it to the hair. In one study, a synthetic cannabinoid receptor agonist (SCRA) was detected in the hair of an individual known to be in rehabilitation and not using drugs. Contamination was suspected, and it was hypothesized that a cap worn

during SCRA use was the source of contamination. Based on this hypothesis, further analyses were conducted. The hair sample was washed, and a part of the baseball cap was washed with methanol. Various SCRAs, particularly MDMB-4en-PINACA, were detected in the hair sample. Similarly, MDMB-4en-PINACA and other SCRAs were found in the cap's washing solution. The concentration of MDMB-4en-PINACA in the hair was 7.9 pg/mg, while in the cap, it was approximately 90 ng/cm². It was concluded that wearing the cap could transfer substances from the cap to the hair, resulting in a false positive (Zschiesche et al., 2024).

Case 3: During doping control analyses conducted on the urine sample of a Dutch professional athlete, the substance norpseudoephedrine was detected. Ephedrine is listed as a prohibited substance by the WADA (TDMK, 2023). For this reason, the detected positivity poses a problem for the athlete. During urine integrity tests, the creatinine concentration was 17.9 mmol/L, indicating that it was not highly concentrated. The athlete denied using performance-enhancing substances, leading to an investigation of the foods consumed. The investigation revealed that the athlete had used a herbal supplement called "Limiet 65 slankheidsdruppels," which was found to contain Ephedra. However, the label on the package stated "evreda," not "Ephedra." Upon analysis, the supplement was found to contain both ephedrine and norpseudoephedrine. Following the recommended usage instructions, three volunteers consumed the supplement, urine samples were collected, and the quantitative analysis of the substances and creatinine levels in the urine samples were examined. Although the norpseudoephedrine concentrations were lower than the 20.2 µg/mL detected in the athlete, the presence of the substance was confirmed. It was demonstrated that the athlete's positive result could have been due to the use of the dietary supplement. After this incident, the Union Cycliste Internationale (UCI) banned this supplement. In summary, it was emphasized that herbal dietary supplements should be carefully examined, product labels may not accurately reflect the actual ingredients, and interpretations should be made accordingly (Ros et al., 1999).

Case 4: Substance transfer through unprotected sexual intercourse is an expected occurrence; however, there is limited literature on this topic. In one study, contamination sources were investigated, and contamination via sexual intercourse was confirmed. Subsequently, seminal fluid was detected in the urine sample of a female athlete. When clinical ejaculate samples were analyzed, the metabolic modulator GW1516 and its metabolites belonging to the PED substance group were found within the seminal fluid. Thus, according to the researchers, the first data on the actual concentration levels were obtained. Considering all these findings, the claim that the transfer of banned substances in sports can occur through unprotected sexual intercourse is strongly supported (Breuer et al., 2024).

Case 5: Couples may exchange oral fluids during kissing, leading not only to the transmission of infectious diseases but also to the transfer of drugs. A recent study demonstrated that up to 5 mL of saliva can be exchanged during kissing, enabling the transfer of substances. In the study involving the anabolic substance Ostarine, a dose of 2.5 µg was detected in a participant who had not used the substance following the reported saliva exchange (Kintz, 2024; Kintz et al., 2024).

Case 6: In a further study by Kintz and Gheddar (2024), the case of an athlete in whom Mesterolone was identified in a biological sample was included. The female athlete challenged the result and initiated legal proceedings. It was hypothesized that the athlete's boyfriend may have transmitted Mesterolone from the anabolic androgenic steroid (AAS) substance group. This is because the athlete's boyfriend is a user of Mesterolone. Hair samples were collected from the athlete and her boyfriend and subsequently analyzed. The athlete's hair was analyzed in 1 cm segments, allowing for the necessary time interval to be accounted for to determine the point at which the urine sample was collected. With a limit of 1 pg/mg, Mesterolone was not detected, while Mesterolone was found in the boyfriend's hair at a concentration level of 70 pg/mg. The arbitration committee was satisfied with the athlete's claim of Mesterolone contamination (Kintz & Gheddar, 2024b).

Case 7: Besides interpersonal transfers, false positives may result from

detecting prohibited substances in sports where clinical drugs are present. This phenomenon has been the subject of several studies (Eichner et al., 2021; Helmlin et al., 2016). A common feature of all cases included in the two studies was that the drugs in question were generic and had been contaminated with diuretic substances. Once it has been established that no intervention has been made on the drug packaging and that a diuretic substance has been detected in the drug, the source of contamination can be identified as the manufacturer or pharmaceutical companies. The contamination may be present in the core, outer coating, or both. In light of these findings, Helmlin et al. (2016) requested that the coating and core be obtained separately from the pharmaceutical company to identify the source of contamination. Subsequent analysis revealed the presence of the diuretic hydrochlorothiazide (HCTZ) in the coating of the drug. In the study conducted by Eichner et al. (2021), a variety of generic clinical drugs contaminated with diuretics, including torasemide and triamterene, particularly HCTZ, were included. HCTZ was the most frequently encountered diuretic in nine different cases. Furthermore, the quantity of diuretic per tablet or capsule and the concentration in the athletes' urine samples were also included. The source of contamination was proven in all these cases; the athletes were not penalized, and the loss of rights was prevented (Eichner et al., 2021).

Case 8: Consumption of plant- and dairy-based foods can result in false positive findings. Several studies have been conducted in this area. Among the prohibited substances with growth promoter properties are anabolic agents such as clenbuterol and β2-agonists like ractopamine and zilpaterol. These substances have been used for many years and can appear in unexpected contexts. For example, zilpaterol is used in some countries, such as Canada and the United States, during fish harvesting, administered before the fish are killed, leading to the substance entering the fish's flesh. Individuals who consume such fish may have positive test results in some instances. To prevent false positives, an experiment was conducted in which volunteers were exposed to zilpaterol-contaminated meat in concentrations that reflect acceptable daily intake

levels. Following the consumption, urine samples were collected, and it was observed that the 5 ng/mL threshold, set as the reporting limit for positive findings, was exceeded (Arcella et al., 2016; Euler et al., 2022; Thevis et al., 2022).

Case 9: Meat contamination with clenbuterol is another issue that requires attention. In 2011, 208 urine samples were collected from athletes during a sports tournament, and clenbuterol was detected in 109 of them. The source of the substance was subsequently investigated, and the meals served to the teams in restaurants were analyzed. Clenbuterol was found in 30% of these meals, which consisted of various meats such as beef, Turkey, and salmon. The concentrations detected in the meals were calculated to be an average of 2.5 μg kg^{-1}. After it was determined that the clenbuterol detected in the athletes was due to consuming contaminated meat, WADA and FIFA ruled that no violation had occurred (Kicman et al., 2016; Thevis et al., 2013). Clenbuterol is administered to animals to promote growth in countries such as China, Portugal, Mexico, and Italy. Consequently, false-positive results may be observed in athletes following the consumption of meat (Kumari et al., 2023).

Certain substances may be present in clinical medications. In such cases, prescriptions should be thoroughly reviewed. Since individuals may misuse clinical drugs, interpretations must be conducted cautiously. Additionally, substances can also be present endogenously within the body. Therefore, a threshold value is established to detect external usage. Values below the threshold are reported as negative, while values above the threshold are considered exogenous and reported as positive (WADA, 2016a).

In Turkiye, the medication Rimobolan, available for therapeutic purposes at pharmacies, contains the active ingredient Methenolone Enanthate. Rimobolan is clinically applied to support patients during recovery or prophylactic against undesired conditions. For example, it is utilized to safeguard women from potential harm in cases of advanced breast cancer (TİTCK, 2023). The active ingredient Methenolone Enanthate is another name for the substance Methenolone (Elks, 2014; Morton & Hall, 1999).

Abroad, synthetic compounds in the form of enanthate are marketed under the trade names Primobolan, which contains Methenolone, and Primobolan-Depot, which is intended for intramuscular injection. It is known that athletes misuse these substances (Kintz et al., 2001). Individuals may similarly abuse Rimobolan as they do with Primobolan abroad.

Some substances may be present in therapeutic drugs while also being produced endogenously within the body. An example of this is nandrolone. Several factors must be considered during urine analyses to ascertain whether the individual used the substance. The detection of nandrolone in urine is indicated by the presence of 19-Norandrosterone (19-NA). In female athletes, low levels of 19-NA may be detected due to pregnancy. Therefore, in cases where nandrolone is detected, it must be demonstrated that the positive result is not attributable to pregnancy if the individual is confirmed to be pregnant (Hemmersbach & Große, 2010).

Another situation involves the use of oral contraceptive medications (birth control pills). 19-NA may be excreted in small concentrations as a minor metabolite of norethisterone, a progestogen agent found in some oral contraceptives that are permitted for use (WADA, 2016b). Urine samples from women who do not use anabolic steroids, including those using oral contraceptive medications containing the progestogen noretisterone, should be carefully evaluated before concluding that the detected concentrations of 19-NA are indicative of anomalous analytical findings (Walker et al., 2009).

Conclusion

All documents obtained during the pre-analytical, analytical, and post-analytical processes are forensic evidence. Athletes use this evidence to defend their rights in cases of victimization. The pre-analytical, analytical, and post-analytical processes must be reviewed to ensure the correct interpretation of the evidence at each analytical stage. It is of paramount importance that forensic toxicologists evaluate false positive and negative results to ensure the accurate reporting of their findings. When making these interpretations, challenges such as polymorphism, metabolism, and dietary habits of individuals should be approached with caution. In this

chapter, sample cases of athletes in and out of competition are presented. It has been clearly seen that the involvement of forensic toxicologists in cases that may result in wrong judgement prevents the loss of individuals' rights.

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EVALUATION OF TECHNIQUES USED IN FINGERPRINT AGE ESTIMATION

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Chapter 6

Evaluation of Techniques Used in Fingerprint Age Estimation

YAKUP GÜLEKÇİ[1], EDA NUR AY[2]

Introduction

Fingerprints, a cornerstone of forensic science, have been used for over 2,000 years to identify individuals for legal or criminal purposes (Barnes, 2011). Beyond identification, another important aspect of fingerprint analysis is determining the age of the fingerprint. The fact that a fingerprint belonging to a person at a crime scene does not necessarily implicate them as the perpetrator, as they may have touched the surface before the crime occurred. Therefore, establishing the time when the fingerprint was deposited on the surface is crucial. After deposition, fingerprints undergo numerous chemical composition and morphological changes. If a universally consistent change that occurs at the same rate across all individuals can be identified, age estimation could potentially be included in routine forensic analyses, bringing significant benefits to the investigative process and the discipline of forensic science overall.

To understand studies focused on ascertaining the age of a fingerprint on a surface, it is first necessary to clearly define what a fingerprint is, identify its components, explore factors affecting aging, and elucidate the changes that occur during the aging process.

Fingerprints as Evidence

The most frequently employed method for individual identification involves the prints formed by the papillary ridges located on the first phalanx of the fingers (Figini, 2012; Houck, 2016). When a hand covered in bodily fluids comes into contact with a surface, these papillary ridges leave fingerprints on the surface (Jones, 2008).

Fingerprints at crime scenes can be classified as visible, latent, and plastic.

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Visible fingerprints can be seen with the naked eye as they are typically stained with blood, dust, paint, or ink. Documenting these prints requires photographing them and then applying various chemicals. Plastic fingerprints, on the other hand, are formed when a finger presses into a malleable substance, leaving an impression. Since these prints are negative in nature, they need to be converted into a positive image after photographing (Figini, 2012; Peixoto & Ramos, 2010). Latent fingerprints, which are the most commonly found at crime scenes, are invisible and require dactyloscopic methods for visualization. Optical techniques are likely to continue for a long time because these methods are non-destructive and easy to use. However, these techniques are often insufficient, and physical or chemical processes may be needed to generate color or luminescence. Selecting the most appropriate fingerprint development method for each case fundamentally depends on two factors and these are the composition of the fingerprint and the surface on which it is deposited (Holder Jr. et al., 2014; Peixoto & Ramos, 2010). The interaction between the fingerprint's composition and the development method enables its visualization. However, the composition of a fingerprint is complex and influenced by internal and external factors (Cadd et al., 2015; Figini, 2012; Houck, 2016).

Eccrine, sebaceous, and apocrine glands secrete the bodily fluids that form fingerprints. These secretions, along with medications, metabolites, and illicit substances used by the individual, contribute to the formation of internal components of the fingerprint (Figini, 2012; Jelly et al., 2009). A fingerprint's intrinsic components consist of 95-99% water and a complex emulsion of organic and inorganic compounds arranged in a three-dimensional matrix (Table 1) (Llewellyn & Dinkins, 1995; Yamashita & French, 2011).

Table 1. Organic and Inorganic Compounds in the Secretory Glands Contributing to Fingerprints (Bleay et al., 2018, 2021; Croxton et al., 2010; Ramotowski, 2001; Yamashita & French, 2011).

Eccrine Gland
Glucose and other reducing sugars, glycogen
Lactic acid and lactate
Peptides (i.e., dermcidin, cathelicidin LL-37)
Proteins (i.e., albumin, cathepsin D, immunoglobulins (IgG, IgA, IgD, IgE))
Pyruvic acid and pyruvate
Urea, uric acid, ammonia
Vitamins (i.e., ascorbic acid, choline, folic acid, niacin, riboflavin)
Metal ions – major (i.e., calcium, iron, potassium, sodium) and trace (e.g., cobalt, copper, lead, magnesium, zinc)
Water
Bromide, chloride, fluoride, iodide, sulfur
Ammonia
Carbohydrates
Sebaceous Gland
Fatty acids (i.e., palmitic, palmitoleic, oleic, stearic, myristic acids)
Fatty acid alkyl esters (i.e., methyl esters of palmitic acid, stearic acid)
Glycerides (i.e., mono-, di-, and triglycerides)
Alcohols
Hydrocarbons (saturated and unsaturated)
Squalene, squalene degradation products (i.e., squalene epoxides, squalene hydroperoxides)
Sterols (i.e., cholesterol)
Sterol esters (i.e., cholesterol esters)
Wax esters (i.e., myristyl myristate, palmityl palmitoleate, stearyl palmitate)
Apocrine Gland
Androgenic steroids
Carbohydrates such as glycogen
Carboxylic acids
Sterols
Proteins

Eccrine glands are distributed throughout the entire body, whereas sebaceous glands are primarily located in areas with a high density of hair follicles, such as the scalp. Sebaceous glands are absent in this region since there are no hair follicles on the palms. However, contact between the hands and other parts of the body can contribute to the formation of fingerprints. Apocrine glands, on the other hand, can be found in the groin, armpits, and genital areas (Becue et al., 2011; Cadd et al., 2015; Croxton et al., 2010; Jelly et al., 2009; Xu et al., 2015).

The external components that contribute to fingerprints include substances such as oils, food contaminants, dust, dirt, blood, cosmetics, moisturizers, gunshot residues, explosives, and illicit substances that a person might come into contact with (Cadd et al., 2015; Champod et al., 2004; Girod et al., 2012; Houck, 2016; Yamashita & French, 2011).

Both internal and external components vary between individuals, as well as across different days or even at different times within the same day when samples are collected (Michalski et al., 2013). Environmental factors also play a role in evaporation, degradation, oxidation, and interactions with microbiological organisms. Pathological conditions further influence the composition (Bleay et al., 2021; Cadd et al., 2015; Houck, 2016).

The Significance of Fingerprint Research

Fingerprints reflect an individual's physical characteristics (such as ridge width, ridge pattern, and minutiae), chemical composition (including water, inorganic and organic compounds), and biological traits (such as gender, health status, age, and nutrition) (Oonk et al., 2018; Wang et al., 2015). In addition, fingerprints provide valuable information about the time elapsed since their deposition on a surface. Estimating the age of fingerprints can offer insights into the timing of a crime. This information is crucial for corroborating witness statements, examining evidence related to the crime, and apprehending suspects (Girod et al., 2014).

The time period during which a fingerprint was deposited on a surface is critically important for investigations. A conviction may hinge on determining

whether the fingerprints discovered at the crime scene were deposited prior to the incident or if they are directly linked to the suspects involved in the crime (Adebisi, 2008; Gardner & Anderson, 2010).

Despite nearly 90 years of research on age estimation from fingerprints, organizations like the Scientific Working Group on Friction Ridge Analysis, Study, and Technology (SWGFAST) have not established an official protocol for this practice (Girod et al., 2016a; Heindl, 1922). Many studies have focused on changes in the bodily secretions that form fingerprints or the morphological evolution of fingerprints over time. Examining the mechanisms of fingerprint formation and the factors influencing fingerprint development is essential to better understanding these studies.

Fingerprint Formation Mechanism and Factors Affecting Fingerprint Development

The composition of a fingerprint is influenced by various factors before deposition, during deposition, and in the subsequent aging process that alters its initial composition (Sears et al., 2012). Before deposition, individual characteristics such as gender, age, race, diet, medications, psychological state, and health play a role. During deposition, conditions like contact duration, contact angle, and pressure are influential, along with surface features like texture and porosity. During the post-deposition aging phase, environmental factors such as humidity, light exposure, dust, air circulation, temperature, UV radiation, and other forms of radiation, as well as the length of time the print remains on the surface, and the use of chemical, physical, or physicochemical fingerprint development methods, all influence the characteristics of the fingerprint (Archer et al., 2005; Cadd et al., 2015; Girod et al., 2012). Therefore, a fingerprint on a surface does not remain the same over time.

Pre-Deposition Variables

Gender: The composition of fingerprints varies between genders. Fatty acids are higher in males, while amino acids are higher in females, with levels in females being approximately twice as high as in males (Brunelle et al., 2018; Croxton et al., 2010). Males tend to have thicker ridges, whereas females have higher ridges (Kralik &

Novotny, 2003; Verma & Agarwal, 2009).

Age: The composition of fingerprints can vary significantly between childhood, adolescence, and adulthood (Figure 1) (Ramotowski, 2001). In children, carboxylic acid salts, esters, and secondary amides are present, but the primary components are lactic acid and sodium salts (Williams et al., 2011), with eccrine compounds being predominant (Mong et al., 2001). During adolescence, the proportion of endogenously synthesized lipids, such as wax esters, squalene, and Δ6 fatty acids, increases, while the proportion of exogenous lipids, including cholesterol, linoleic acid, and Δ9 fatty acids, decreases (Girod et al., 2012). After adolescence, the variability in fingerprint composition slows down (Ramotowski, 2001). Unlike adults, children's fingerprints contain high amounts of cholesterol (Downing et al., 1977). In adults, wax esters and triglycerides are predominant (Antoine et al., 2010). Due to the higher molecular weight and lower volatility of fatty acids in adults, children's fingerprints fade much more quickly (Antoine et al., 2010; Ramotowski, 2001). This difference allows for the differentiation between child and adult fingerprints and leads to variations in coloration after dusting methods are applied (Antoine et al., 2010).

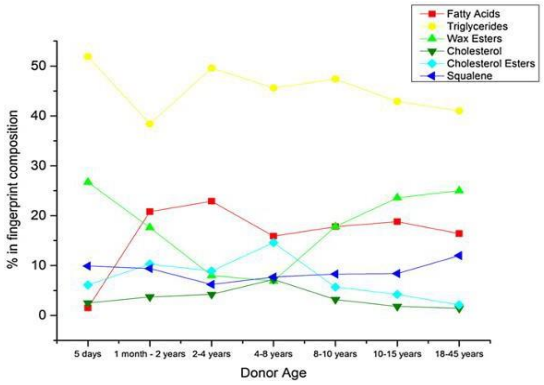

Figure 1. Age-related changes in fingerprint components *(Ramotowski, 2001).*

Health: Various skin conditions, such as acne, and the active ingredients in medications used to treat these conditions can alter the secretion levels of the sweat glands (Ganceviciene & Zouboulis, 2010). For instance, medications containing isotretinoin, commonly used in acne treatment, reduce sebum production and the size of sebaceous glands (Dalziel vd., 1987). A study involving patients treated with isotretinoin found a decrease in the percentage of squalene and wax esters (Strauss

& Stranieri, 1982). Reduced sweating in response to pharmacological or thermal stimuli is termed hypohidrosis (Cheshire & Fealey, 2008). Hypohidrosis, which can be a symptom of conditions like hypohidrotic ectodermal disorder, ectodermal dysplasia, and Fabry disease, results in decreased sweat secretion from eccrine glands (Cluzeau et al., 2011; Goleva et al., 2020; Motamed et al., 2006). In atopic dermatitis, changes in sebaceous glands lead to a reduction in squalene, wax esters, and total lipids, along with an increase in cholesterol (Shi et al., 2015). During pregnancy, hormonal fluctuations affect the function of secretory glands, altering secretion levels (Braude & Hamilton-Fairley, 2008; Hiller-Sturmhöfel & Bartke, 1998). From the eighth week of pregnancy, cholesterol levels increase; between the sixth and ninth months, sebaceous gland secretions increase while apocrine gland secretions decrease, and between the third and sixth months, eccrine gland activity rises (Akdağ, 2012; Burton et al., 1970; Kar et al., 2012; Martin & Leal–Khouri, 1992). A reduction in the function of any of the three primary glands responsible for fingerprint formation can adversely affect fingerprint development.

Variables During Deposition

The contact area, contact angle, force applied to the surface, deformation, and finger sliding along the surface all influence fingerprint formation (Maceo, 2009). Greater pressure results in more residue being transferred to the surface; however, extending the duration of contact does not significantly change the number of cells transferred(Balogh et al.., 2003; Jasuja et al., 2009).

Surface structure: Surfaces are categorized as porous, semi-porous, and nonporous. On porous surfaces, adhesion forces between the bodily secretions forming the fingerprint and the surface enable the components to penetrate the surface (Almog et al., 2004). This penetration helps protect the fingerprint from degrading environmental conditions (Girod et al., 2016a). Eccrine components are absorbed more rapidly than sebaceous ones on porous surfaces (Almog et al., 2004; Girod et al., 2012). Reagents that react with amino acids are preferred for such surfaces because they remain stable once absorbed (Holder Jr. et al.,

2014). Eccrine substances are absorbed more slowly on semi-porous surfaces than on porous surfaces. When comparing the absorption rates of sebaceous and eccrine components, eccrine substances are still absorbed more quickly (Almog et al., 2004; Girod et al., 2012). Nonporous surfaces, which are frequently encountered at crime scenes, do not allow fingerprint secretions to penetrate. As a result, these secretions remain on the surface, making them more vulnerable to environmental factors and more prone to damage (Siegel et al., 2013; Velho et al., 2017).

Contaminants: The composition of a fingerprint can vary based on the cleanliness of the individual's hands. Factors such as whether the hands are washed, dirty, or have lotion applied can affect the composition of the fingerprint. Additionally, contaminants on the hands, such as blood, dust, explosives, or gunshot residues, can influence the fingerprint (Czech et al., 2019).

Variables in the Aging Stage

Light: Exposure to light causes variations in the components of a fingerprint. Squalene and fatty acids degrade more rapidly under light. Despite being present at very low levels in newly formed fingerprints, squalene can still be detected after 33 days in dark conditions, whereas it is undetectable after 9 days under light exposure. Short-chain fatty acids form and saturated fatty acids initially increase before decreasing (Archer et al., 2005; Jones et al., 2001). The ozonolysis rate of triacylglycerol in a fingerprint exposed to light increases significantly compared to one stored in a closed environment (Hinners et al., 2020). For a fingerprint stored in dark conditions, the monounsaturated C18 fatty acid concentration initially increased before decreasing (Archer et al., 2005).

Temperature: There is a direct correlation between temperature and the aging process. Volatile components in fingerprints evaporate more quickly as the temperature rises (Bleay et al., 2018). Unlike light-induced degradation, amino acids, which are abundant in eccrine secretions, have been found to undergo temperature-induced degradation (De Paoli et al., 2010). Triglycerides, frequently studied in fingerprint aging research, thermally decompose into a series of compounds,

such as alkanes, alkenes, alkadienes, aromatics, and carboxylic acids (Srivastava & Prasad, 2000). A drop in ambient temperature also affects fingerprint quality. A study examining fingerprints on surfaces using optical sensors found that the quality of fingerprint images deteriorated when the surrounding temperature fell below freezing, likely due to the increased dryness of the skin (Kang et al., 2003).

Humidity: At low humidity levels, the fluid in the fingerprint may dry more rapidly. In contrast, the water in the fingerprint fluid persists for longer at higher humidity levels. High humidity plays a significant role in migrating water-soluble components in fingerprints deposited on the porous surfaces. When the surface absorbs more moisture, components such as urea and sodium chloride can shift from their initial locations, resulting in blurred or diffuse fingerprints (Bleay et al., 2018).

Contaminants: Understanding the degradation of contaminants in fingerprints is crucial. For instance, the water components in blood evaporate within hours or days, drying out the fingerprint, whereas proteins degrade over several years. Contaminants such as grease tend to dry and degrade gradually (CAST, 2014).

Ultraviolet (UV) light: UV light affects fingerprint components more than visible light. When exposed to UV light, squalene degrades into formaldehyde and malonaldehyde, with this conversion happening faster at shorter wavelengths (Yeo & Shibamoto, 1992). Eccrine components are less affected by UV light. However, lactic acid has been found to undergo photochemical reactions upon exposure to simulated sunlight, while urea and amino acids remain unaffected under the same conditions (De Paoli et al., 2010).

Bacteria: Skin flora from various species can accumulate on a surface after a finger touches it (Phan et al., 2020). Under specific storage conditions, bacteria can persist in fingerprint residues and continue to alter the composition through enzymatic activity (Cadd et al., 2015; Pleik et al., 2016). For example, facultative anaerobes are known to hydrolyze sebaceous triglycerides into diglycerides, monoglycerides, and free fatty acids within the sebaceous gland

ducts (Byrd et al., 2018; Goetz et al., 1984).

Anaerobic conditions facilitate the hydrogenation process, which converts unsaturated bonds and increases the levels of saturated fatty acids (Srivastava & Prasad, 2000). The reduction in saturated fatty acid levels is primarily attributed to evaporation and degradation (Archer et al., 2005; Cadd et al., 2015), while the degradation of unsaturated fatty acids is likely due to oxidation (Pleik et al., 2016).

Fingerprint Development Reagents: Fingerprints obtained from crime scenes are first analyzed using various development reagents. Once the prints become visible, they are identified to determine the individual's connection to the crime. Although the routine application of fingerprint age estimation is not yet common, the existing fingerprint can be evaluated for identification purposes if required by the court. It is, therefore, essential to comprehend how chemicals impact the aging process of fingerprints (Cadd et al., 2015). Additionally, the fingerprint development chemicals should not damage the equipment used for age determination (Koenig et al., 2011).

The components in body fluids that form fingerprints react with various chemicals, which may have different properties. For example, Oil Red O and physical developers react with lipids and are more effective at developing older fingerprints (Salama et al., 2008). Research indicates that fingerprint development chemicals interacting with amino acids are effective with older prints, suggesting that amino acids remain relatively stable over time (Hansen, 2005).

A study examining the effects of fingerprint development reagents on the morphology and composition of aged fingerprints analyzed the impact of magnetic powder, indandione, and cyanoacrylate on squalene, cholesterol, and myristyl myristate. While magnetic powder contaminates the column, formulations created using cyanoacrylate and HFE-7100 (the main solvent in indandione) do not pose a problem. However, formulations made with dichloromethane significantly reduced the recovered cholesterol and squalene in fingerprint residues (Koenig et al., 2011).

Duration of Fingerprint Presence on the Surface: As a fingerprint remains on a surface, its volatile components, moisture, and oily substances gradually evaporate (Sodhi & Kaur, 2001). Over time, the fingerprint becomes more viscous, and its thickness changes (Champod et al., 2004; CAST, 2014). As water evaporates, organic and inorganic compounds accumulate in a waxy layer, reducing the surface area that needs to interact with fingerprint development chemicals, making the reaction more difficult (Mong et al., 1999). Within just 72 hours of being deposited, eccrine components in the fingerprint can decrease by as much as 98% of their original mass (Daluz, 2014). Organic compounds, amino acids, and inorganic salts remain as the primary components. These compounds exhibit much greater stability and tend to persist for longer periods, provided they are not exposed to moisture (CAST, 2014).

Physical Environment: When fingerprints are deposited on surfaces exposed to heavy rain or submerged in water, the water-soluble components may be completely removed from the fingerprint residues (Bleay & de Puit, 2018).

Airflow: Wind can dry surfaces in outdoor environments by moving air across them. However, it can also carry solid particles or liquid droplets that may interact with fingerprint residues. In indoor settings, airflow is easier to regulate. Devices like fans and air conditioners, which generate relatively high airflow rates, can significantly impact the composition of fingerprint residues (Bleay & de Puit, 2018).

Variables in Determining the Age of Fingerprints

Once deposited on a surface, fingerprints are exposed to numerous variables. Factors such as humidity, temperature, and light are unlikely to remain constant in real-world situations (Francese & Bradshaw, 2021). Therefore, studies conducted under controlled conditions often yield limited results. If there is any change in a variable, the age of the fingerprint must be recalculated (Merkel et al., 2011; Poletti et al., 2021). The content of fingerprints can vary between individuals or even at different times for the same individual. Since the initial quantities of chemicals at the deposition

time are unknown, studies focusing solely on changes in quantity may lack depth (Boseley et al., 2022; Koenig et al., 2011). There are differences between the initial state of the fingerprint on the surface and its condition at the time of analysis. It is anticipated that by understanding the gradual changes in the factors causing these differences, the age of the fingerprint can be estimated.

Research on this subject primarily focuses on the physical changes in fingerprints, alterations in chemical composition, and the impact of environmental conditions (Wertheim, 2003). For the first two methods to be applied, the conditions present at the time of fingerprint deposition need to be recreated. However, if a common value or ratio is found in all fingerprints created under the third method, calculations would be easier than the other methods (Beardsmore-Rust et al., 2009; Wertheim, 2003).

Techniques for Determining the Age of Fingerprints

Numerous experiments have been conducted to determine the age of fingerprints. These experiments can generally be categorized into two main approaches: changes in the chemical composition of fingerprint components and variations in their physical characteristics.

Techniques For Determining Fingerprint Age Based on Physical Characteristics

Fingerprints are primarily used as visual data for identification purposes. However, beyond identification, the physical properties of fingerprints can also be utilized as parameters for aging. Techniques for fingerprint age determination based on physical characteristics have been developed using morphological changes in ridges, valleys, minutiae, and pores (De Alcaraz-Fossoul et al., 2019b); topography and contrast differences created on the surface (Merkel et al., 2012); optical properties such as fluorescence emission (van Dam et al., 2014); reactions with fingerprint development powders (Colella et al., 2020); and electrical-based approaches (Watson et al., 2011). Although these analytical methods are relatively simple and cost-effective, they may not always provide an objective assessment.

Morphological Techniques: When fingerprints persist on a surface for an

extended period, there is a notable reduction occurs in their components, such as water, accompanied by morphological changes in the ridges (Barros et al., 2013). These morphological characteristics encompass two-dimensional features like ridge width, ridge continuity, minutiae count, and pore size. Furthermore, three-dimensional features like ridge height and ridge volume can also be examined (De Alcaraz-Fossoul et al., 2019b). Since fingerprints have a three-dimensional structure, two-dimensional analyses may be insufficient (Chen et al., 2021).

Morphometry has the potential as a measurement method for identifying fingerprint aging patterns (Barros et al., 2013). This technique identifies morphological characteristics using microscopy, photography, or scanning techniques and then quantifies them through visual statistical processing. This allows for the simultaneous capturing of morphological images and fingerprint-aging information (Rosa et al., 2017). Visual techniques establish a direct relationship between the physical appearance of the fingerprint and its age. The cost of equipment and consumables is low, the method is non-destructive compared to chemical analysis, and the fingerprints can be used for further analysis after examination (De Alcaraz-Fossoul et al., 2019b).

A study examining ridge widths found that fingerprints deposited on glass are more resistant to degradation than those deposited on plastic. Ridge width loss became noticeable within the first 60 days (Figure 2). Although the overall degradation pattern appeared similar, variations were observed between participants, different fingers of the same participant, and between different surfaces (De Alcaraz-Fossoul et al., 2019a).

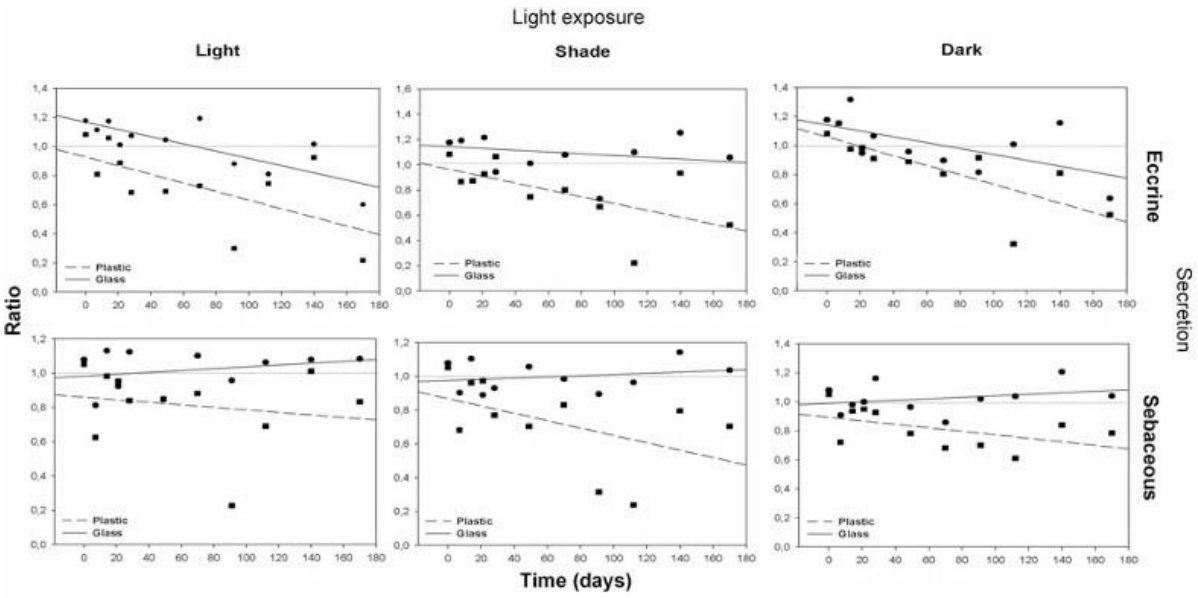

Figure 2. The effect of different secretion types and light properties on fingerprint aging (De Alcaraz-Fossoul et al., 2019a).

Popa and colleagues (2010), in their 180-day observation, demonstrated that characteristics such as inter-pore distance, ridge and valley width, pore presence, and ridge thickness exhibited different levels of change in indoor and outdoor environments. Additionally, the number of minutiae decreased over time.

Using atomic force microscopy, ridge expansion, forming interconnected islands, was detected over time. In a study where fingerprints were placed on polished silicon and Formica surfaces for two months, a reduction in ridge height and a spreading of components across the surface were observed. The texture and scratched nature of the surface influenced the spread of the fingerprint (Popov et al., 2017).

The gradual disappearance of sebum-rich fingerprints on different surfaces was examined using phase contrast microscopy. While the prints on polypropylene and polyvinyl chloride disappeared within four days, those on glass remained visible for up to 58 days. On polypropylene, the ridge lines appeared as droplets between days 4 and 58, while on polyvinyl surfaces, the ridge lines lost their definition after four days. A limitation of the study is that it involved only one participant (Moret et al., 2015).

Over three months, ridge height changes in fingerprints deposited on

slides were examined using light interference microscopy. Utilizing phase shift and refractive index, the study showed that the cross-sectional profile of the droplet forming the fingerprint changed under various environmental factors and viscosity alterations. Maximum droplet thickness and lateral dimensions decreased over time. Due to chemical inhomogeneity, the variable drying rate within the droplet was suggested as a possible cause of the irregular topography observed over time after deposition. The initial major change in topography was attributed to the evaporation of more volatile components. The absence of a correlation between drying rate and ambient humidity suggests a low water concentration at the droplet surface (Thomas & Reynoldson, 1975).

Optical Techniques: The primary fluorescence source in fingerprints is the amino acid tryptophan. It has been proposed that the gradual degradation of tryptophan fluorescence and its fluorescent derivatives—such as indole acetic acid, harman, norharman, and xanthurenic acid—can serve as a method to estimate the time elapsed since a fingerprint was deposited on a surface. In a three-week study, thin-layer chromatography coupled with fluorescence spectroscopy was employed to monitor this process. Each compound has distinct emission and absorption spectra. As tryptophan decomposes and the ratio between the amino acid and its derivatives shifts, there is a corresponding change in the composite fluorescence emission peak, which may be linked to the fingerprint's age (van Dam et al., 2014).

In another study, time-resolved spectroscopy with an ultraviolet-pulsed laser was used to measure aged fingerprints stored under various conditions. Fingerprints exposed to sunlight or fluorescent light deteriorated more rapidly compared to those stored in darkness. Additionally, higher humidity levels accelerated the rate of degradation. A decline in fluorescence intensity was observed over time. As the storage period extended, a new fluorescence emission peak at a longer wavelength (440 nm) appeared, which was absent in earlier spectra. This peak was utilized to track the aging process of the fingerprint, suggesting that the 440 nm peak could be a marker for the time elapsed since the fingerprint was deposited (Akiba et al., 2018).

An optical chromatic white light sensor based on chromatic aberration contrasts the fingerprint and the surface, while the three-dimensional topography mode allows for height measurement. In a 24-hour observation period, the contrast decreased as the fingerprint aged. The analyzed fingerprints could be categorized into two groups: those deposited for 0-5 hours and those for 5-24 hours. Various factors affecting the aging parameter were also examined in the study. While sweat composition, humidity, temperature, UV exposure, wind, surface type, contaminants, scanning resolution, and measured area size had significant effects, contact duration and contact pressure were insignificant. Although this is a non-contact method, it can only be applied to clean, smooth surfaces (Merkel et al., 2012).

Dusting Method: The dusting method is frequently used to make latent fingerprints visible at crime scenes. Due to its extensive use, researchers have been motivated to study how dusting parameters influence fingerprint aging. Over time, residues transferred by the fingerprint to the surface may evaporate or oxidize, reducing the amount of dust that can adhere to the ridges. Degradation of chemical components lowers dust affinity (Dorakumbura et al., 2016; Popov et al., 2017).

In a study related to a burglary case, where it was claimed that the fingerprint found on a lamp was left before the incident, fingerprints were deposited on the top, middle, and bottom of the bulb and developed at different intervals using dusting. When the quality of fingerprints lifted with adhesive tape was assessed, no correlation was found between the decrease in fingerprint quality score and the time the print remained on the heated bulb (Colella et al., 2020).

Eccrine and sebaceous-rich fingerprints aged under both dark and light conditions were made visible using titanium dioxide-based powder. Similar degradation patterns were observed in both conditions. However, the brush used for applying the powder can damage the ridge lines, as it directly touches the ridges. Sebaceous-rich fingerprints on glass surfaces remained intact for over six months (De Alcaraz-Fossoul et al., 2013).

Sebaceous-rich fingerprints created on ceramic and glass surfaces were dusted

with titanium dioxide or carbon and compared in terms of color contrast. It was found that color contrast was not affected by gender, individual differences, type of dust, or surface type. A study that analyzed color histograms for statistical evaluation suggested that this technique could be used for age estimation due to its consistent results across different individuals and at different times (De Alcaraz-Fossoul et al., 2021).

Electrical Techniques: Electrostatic charge patterns of fingerprints on insulated surfaces such as PVC, PTFE, PVDF, and acetate were monitored over a 14-day period and visualized using an electrostatic potential sensor (EPS) (Watson et al., 2011). This technique allows for imaging static charge distribution without damaging the fingerprint, offering a non-contact and cost-effective solution (Watson et al., 2011). However, this method encounters challenges such as extended scanning times, limited detection areas, and constraints related to the thickness of the insulating surface (Lewis et al., 2010). The technique posits that the degradation of the fingerprint's charge is affected exclusively by the physical properties of the material and environmental factors. Studies indicated that while the overall charge level diminished over time, image resolution was still achievable. Moreover, it was suggested that although very old fingerprints might not be detectable through charge imaging, age estimation could be feasible for recently deposited fingerprints (Watson et al., 2011).

An innovative approach has been explored to understand the aging processes of fingerprints deposited on metallic surfaces. In this sense, Electrochemical impedance spectroscopy (EIS) was utilized to examine both the fingerprint residues and the underlying metallic surface, addressing their electrochemical interactions for the first time (Rosa et al., 2017).

Additionally, PeakForce quantitative nanomechanical mapping (PF QNM) atomic force microscopy (AFM) was employed to explore the adhesion and topographical changes of the droplets that form surface fingerprints. The variability in adhesion observed within a single fingerprint droplet suggests heterogeneity at the nanoscale. Real-time imaging of material transfer from

ridge to valley underscores the device's sensitivity. It was also observed that the adhesion properties of the droplets evolved as the fingerprints aged (Dorakumbura et al., 2016).

Fingerprint Age Determination Techniques Regarding Chemical Composition Characteristics

Fingerprint identification features can be compromised in cases where partial or smudged prints are present, the applied visualization technique is insufficient, resulting in poor image quality, or the suspect's fingerprint records are not found in databases. In such situations, technologies that can provide additional information from the endogenous and exogenous chemical content of fingerprints come into play. One of these additional pieces of information is when the fingerprint has been present on the surface (Francese et al., 2013).

Spectroscopic Techniques: Fourier Transform Infrared (FTIR) microspectroscopy is a technique frequently used to characterize the components of fingerprints. Changes in the infrared spectrum can be detected in real-time (Fritz et al., 2013; Johnston & Rogers, 2018). The alterations in fingerprints left at different temperature conditions, up to 75 °C, for a duration of 5 hours were examined using FTIR. After 5 hours at high temperatures, the amount of sebaceous compounds decreased significantly, while at temperatures below 45 °C, the compounds changed very little. An increase in the OH stretching band (at 3250 cm^{-1}) was observed over 5 hours at all temperatures, indicating that various oxidation processes were occurring. Unsaturated lipids increased the OH stretching band, whereas saturated compounds exhibited no change (Johnston & Rogers, 2017).

A study utilizing FTIR was conducted to investigate the intermolecular interactions of the primary components in natural fingerprints. Analog samples were prepared by pipetting the main constituents of sebaceous- and eccrine-rich fingerprints onto microscope slides. The findings revealed that the absence of squalene and cholesterol significantly limited the interactions among other organic compounds in these analog samples. These interactions are believed to provide insights into the aging processes that occur following

fingerprint deposition (Johnston & Rogers, 2018).

Raman spectroscopy is advantageous because it allows the examination of solid, liquid, and gaseous substances without causing damage and requires no pre-treatment. Its high sensitivity enables the measurement of very low quantities (Chen, 2020). In a study examining proteins, carotenoids, squalene, and unsaturated fatty acids in fingerprints using Raman spectroscopy under different light conditions, clear differences were observed between fresh fingerprints and those aged for one month. Proteins remained relatively stable under light exposure, while carotenoids were the most affected components. Some signals attributed to squalene and fatty acids decreased for one month. However, due to high variability between measurements, it was concluded that careful interpretation using sufficient repeat analyses is necessary (Andersson et al., 2017).

Laser-induced breakdown Spectroscopy (LIBS) has been used to determine fingerprint deposition time. In an analysis conducted by dividing one hour into ten-minute intervals, an increase in the CN bond was observed over time. Environmental effects such as drying, dehydration, and degradation increased the CN band in fingerprints. By basing the analysis on this increase, fingerprints deposited at different times could be distinguished using LIBS and Raman spectroscopy combined with SIMCA and PLS-DA classification methods (Yang & Yoh, 2018).

Liquid Chromatography-Mass Spectrometry (LC-MS) was employed to identify unsaturated triglycerides and their natural degradation products in order to understand the fingerprint aging process. The degradation of unsaturated triglycerides differs between open and closed environments. The study indicated that the ratio of TG(48:1) to TG(48:0), TG(48:0)-monooxonide, and TG(48:0) could serve as parameters for estimating fingerprint age. However, due to the presence of other compounds such as squalene in fingerprint residues, the degradation rates of triglycerides in fingerprints cannot be directly compared to those of reference lipids (Pleik et al., 2018).

In addition to chemical changes in fingerprints, some studies have focused

on molecular diffusion. In one such study, the diffusion of palmitic acid was investigated using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). For fingerprints deposited on silicon wafers, a formula was derived in hours that could determine the location of palmitic acid. It is suggested that age determination is accurate up to 96 hours; however, since molecules will not remain the same over longer periods—they will break down or oxidize—the method is not practical for extended durations (Chen et al., 2021; Muramoto & Sisco, 2015).

Chromatographic Techniques: Francese et al. (2013) conducted a review of mass spectrometry imaging methods for fingerprint visualization. The study highlighted the challenges and complexities of the age determination process. Evaluating all parameters affecting fingerprint age in a single study is extremely difficult. Moreover, the common use of sebaceous-rich fingerprints in most studies does not reflect real-world cases.

Mass spectrometry has significantly contributed to understanding the molecular composition changes in fingerprints over time, especially concerning lipids. Combining chromatography with mass spectrometry has allowed for analysis of the complexity of molecular lipid composition over time for both volatile and non-volatile species. However, these approaches require the extraction of molecular fingerprint components and are destructive (Francese & Bradshaw, 2021).

Lipids are commonly used in age determination because they are more stable than other components. Gas Chromatography-Mass Spectrometry (GC-MS) facilitates the comparative application of targeted lipids in fingerprints. Out of thirteen types of lipids, six—including isopropyl dodecanoate, cholesterol, squalene, and its derivatives—were selected. Unknown effects (e.g., deposition time, pressure, temperature, and light conditions) were then investigated using statistical methods such as Principal Component Analysis (PCA), univariate exponential regression, and Partial Least Squares (PLS) regression (Figure 3). While PLS regression showed significant deviations, exponential regression was the method capable of providing differentiation

across all parameters. When storage conditions were known, the age of all test fingerprints was accurately assessed; however, when these conditions were unknown, the results were inconclusive (Girod et al., 2016b).

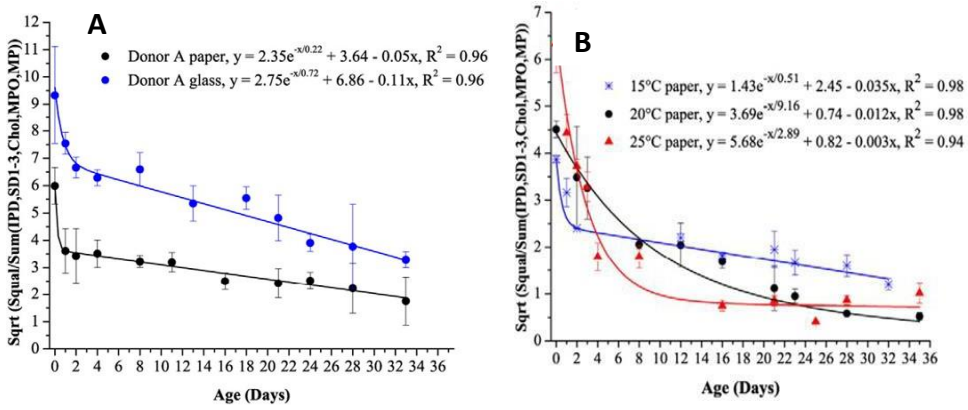

Figure 3. Exponential linear regression images of fingerprints deposited on microfiber filters and aged on different surfaces (A) and at different temperatures (B) *(Girod et al., 2016b).*

The ratio of squalene to cholesterol analyzed using GC-MS showed that the relative standard deviation of the ratio of these two chemicals (less than 20%) was lower than the standard deviation of the individual compounds (up to 80%). The study also emphasized the importance of the type of surface. While squalene exhibited lower values on glass surfaces, it showed higher values on microfilters (Weyermann et al., 2011). O'Hagan and Green found that this is because samples on glass are collected by swabbing, whereas microfilters are directly immersed in the extraction solvent (O'Hagan & Green, 2018). On glass surfaces, squalene began to decrease on the first day and became undetectable after the first week, whereas it could be detected on microfilters for up to 30 days. Cholesterol decreased much more slowly on glass and showed no changes on microfilters during the examined time period (Weyermann et al., 2011).

Serine is the most abundant amino acid in adult fingerprints (Birnbaum, 2011; Croxton et al., 2010) and can be detected by GC-MS at temperatures up to 150 °C (Croxton et al., 2010). Free fatty acid components like oleic acid and aspartic acid have more complex side chains compared to amino acids. These molecules can withstand temperatures

up to 250 °C, depending on the duration of exposure (Birnbaum, 2011).

The fatty acid profile of sebaceous-rich fingerprints was characterized using gas chromatography-flame ionization detection (GC-FID). Additionally, the effects of methods such as dusting, iodine fuming, and silver nitrate were evaluated over a 30-day aging period under controlled temperature, photoperiod, and humidity parameters. The results showed that myristic acid, palmitic acid, stearic acid, and oleic acid changed statistically significantly during the 30-day analysis period. The decrease in myristic acid can be explained by evaporation, while changes in stearic acid and palmitic acid may result from shortening long-chain fatty acids. An increase in the concentration of saturated fatty acids was observed in the early days, returning to previous levels toward the end of the period. For unsaturated fatty acids, a decrease was noted over time as double bonds converted to saturated bonds. However, the study was conducted with a single participant (Poletti et al., 2021).

Proteogenomic methods that utilize protein sequence variations arising from DNA mutations have recently begun to be used in forensic science studies. Some research aims to co-extract samples left on surfaces by touch for both protein and DNA analyses. Schulte and colleagues addressed this issue in their study. They enabled both classical DNA analysis and proteolytic digestion, followed by LC-MS analysis on the same touch sample. The analyses showed that the amount and quality of proteins remained constant regardless of time and individual repetitions (Schulte et al., 2021).

Enantiomers of various amino acids found in fingerprints (threonine, alanine, histidine, serine, valine, proline, methionine) were separated and quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The conversion of the L-stereoisomer of amino acids to the D-stereoisomer was investigated. Fingerprints deposited on glass were stored in the dark at room temperature for six months. Only D-serine showed a significant change. An increase was observed with aging up to 30 days for all participants. Beyond this period, three participants showed further increases, while the other three

exhibited stability followed by a decrease (van Helmond et al., 2020).

Proteomic-based LC-MS analysis is a new methodology developed to estimate the age of fingerprints. In the study, protein profiles of fingerprints were extracted, and the potential effects of hand contact with body fluids on age biomarkers were examined by simulating real case studies. Analyses conducted over 16 days concluded that proteins such as K2C1, Keratin type II cytoskeletal 2 epidermal (K22E), Keratin type I cytoskeletal 9 (K1C9), K1C10, and DCD could be used as aging parameters. After eight days, all proteins except DCD showed an increase, while DCD decreased. Proteins from body fluids did not have a negative effect on aging parameters (Oonk et al., 2018).

The glyceride fraction has various structures that complicate its identification, tracking of degradation pathways, and identification of products. In a study examining changes in diglycerides and triglycerides using ultra-performance liquid chromatography–ion mobility separation–quadrupole time-of-flight mass spectrometry (UPLC-IMS-QToF-MS), it was observed that unsaturated triglycerides oxidized to mono-ozonides and di-ozonides (Figure 4). However, the amount of ozonide initially deposited on the surface by the participant is unknown (Frick et al., 2020). Ozonolysis, involving one of the most reactive oxygen species in the air, is a primary lipid degradation pathway for the components forming fingerprints (Pleik et al., 2018). Exposure to glycerides to air is more effective in reducing degradation than exposure to light. Triglyceride aldehyde derivatives were found to be higher on the 28th day compared to the first day (Frick et al., 2020).

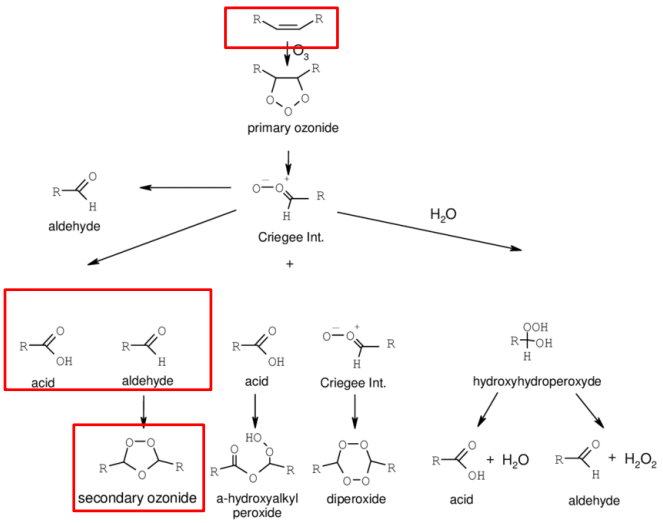

Figure 4. Reaction mechanisms of unsaturated triglycerides with ozone. Structures marked in red are those observed in the relevant study *(Frick et al., 2020; Vesna et al.,2008).*

The age of fingerprints has been determined by examining the diffusion of various compounds from the ridges into the furrows using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) imaging. Fatty acids and triacylglycerols were analyzed in both fresh and aged fingerprints on four different surfaces. It was initially expected that higher molecular weight triacylglycerols would diffuse more slowly than fatty acids, making it possible to identify older fingerprints. However, the study revealed that interactions between endogenous compounds and the surface had a much greater impact on diffusion than molecular weight. A qualitative correlation was observed between the surface's hydrophilicity and/or hydrophobicity and the diffusion of fingerprint compounds, offering valuable insights for creating a diffusion model for fingerprint aging (O'Neill & Lee, 2018). The National Institute of Standards and Technology has also found that biomolecules such as fatty acids consistently and predictably move from the ridges into the furrows (van Dam et al., 2016).

Future Directions

Despite numerous studies aimed at determining the age of fingerprints, a definitive method has yet to be established. Based on current research, the characteristics and conditions that the future device and the analyzable

properties should possess are as follows:

The chemical composition of the fingerprint component to be analyzed must be clearly defined. Intermediate and final products of reactions catalyzed by environmental factors such as heat and light should be identified. The ratios between primary components, intermediates, and final products should be measured at specific time intervals. Studies that utilize the ratios of different components are more successful than those focusing on a single component because factors such as surface type, pressure, and contact duration introduce variability in the amount of chemical composition transferred to the surface (Girod et al., 2016b; van Dam et al., 2016). Optimal conditions under which the content remains stable should be identified, and the evidence should be stored in laboratory conditions (Hinners et al., 2020).

The biomarker must not overlap with substances present as contaminants on the finger. The technique employed must distinguish between contaminants and fingerprint components. Since most fingerprints undergo various processes for identification purposes, the biomarker must remain unaffected by these processes. Additionally, the method used should not damage the appearance of the fingerprint (De Alcaraz-Fossoul et al., 2013).

Numerous factors can cause changes in fingerprint chemistry. These should be examined in detail, and if possible, a component that shows minimal variation among individuals should be selected. The chosen component must be present in every individual and be unaffected by personal characteristics such as age, gender, and race (Bleay et al., 2018). Changes in the component due to special conditions such as disease should also be investigated. Moreover, the analysis method should be suitable for fingerprints on different surfaces. The device used for analysis should be accessible, affordable, fast, environmentally friendly, and possess adequate specificity. Studies conducted to develop the method should include sufficient participants and repetitions. The data obtained from the analysis should be interpreted objectively (Table 2) (Cadd et al., 2015; Girod et al., 2016a; Koenig et al., 2011).

Conclusion

Various studies aimed at determining how long fingerprints remain on surfaces have examined the compositional changes of fingerprints before deposition, during deposition, and after aging over time. These studies have thoroughly evaluated the variables affecting the persistence of fingerprints, such as environmental conditions and individual factors. They have focused on the detection methods of various chemical substances and morphological features in the bodily fluids that form fingerprints (Bleay et al., 2018).

Studies that rely on the temporal changes of a single chemical require knowledge of the initial composition to determine the starting point (Cadd et al., 2015). In contrast, studies that assess the ratio of different chemicals to each other or the ratio between primary substances and their products promise more successful results, as they do not depend on knowing the initial quantity of the substance (Weyermann et al., 2011). Despite the examination of numerous analytical techniques and fingerprint components, a definitive method has yet to be established. Age estimation of fingerprints, which remains an area for development in forensic sciences, is a promising research field that could significantly contribute to solving cases once incorporated into routine analyses.

Table 2. *Summary of Techniques Used for Fingerprint Age Estimation*

References	Analysis Method	Aging Parameter	Aging Duration	Advantage	Disadvantage
(De Alcaraz-Fossoul et al., 2019a)	Photography	Ridge Widths	6 months	Easy Analysis Low Cost	There are variations between participants, different fingers of the same participant, and across surfaces.
(Popa et al.., 2010)	Photography, Microscopy	Distance Between Pores, Ridge and Furrow Widths, Number of Minutiae	3 months	High Resolution Non-destructive to Sample	It can only be applied to smooth surfaces
(Popov et al.., 2017)	Atomic Force Microscopy	Reduction in Ridge Height, Lateral Migration of Components	2 months	Topography is Created 2D and 3D Analysis	Surface texture variations affect the results
(Moret et al.., 2015)	Phase Contrast Microscopy	Ridge Appearance	2 months	Non-destructive to Sample	Small sample size.
(Thomas & Reynoldson, 1975)	Transmitted Light Interference Microscopy	Ridge Height	3 months	Refractive Indices are Used	Chemical inhomogeneity affects results.
(van Dam et al.., 2014)	Thin Layer Chromatography Combined with Fluorescence Spectroscopy	Ratio Between Amino Acids and Their Derivatives	3 weeks	Stronger Fluorescence in Aged Fingerprints	Provides information only on fluorescent substances. Qualitative data.
(Akiba et al.., 2018)	Time-Resolved Spectroscopy	Fluorescence Emission at 440 nm	1 year	Can Distinguish Differences in Various Storage Conditions	Small sample size.
(Merkel et al.., 2012)	Chromatic White Light Sensor	Contrast and Ridge Height	0-5 hours, 5-24 hours	Topography is Created 2D and 3D Analysis	Applicable only on clean and smooth surfaces.
(Colella et al.., 2020)	Dusting	Fingerprint Quality Score	672 hours	Easy Analysis Low Cost	No correlation found between fingerprint quality score and age. Dusting can damage the sample.
(Watson et al.., 2011)	Electrostatic Potential Sensor	Electrostatic Charge Pattern	14 days	Non-destructive to Sample High Resolution	Long analysis time. Effective only on thin surfaces. Small detection area.
(Rosa et al.., 2017)	Electrochemical Impedance Spectroscopy	Capacitance Distortion	45 days	Low Noise, High Sensitivity	It is suitable only for metallic surfaces. It is affected by surface roughness.

Table 2. *(Continued) Summary of Techniques Used for Fingerprint Age Estimation*

References	Analysis Method	Aging Parameter	Aging Duration	Advantage	Disadvantage
(Johnston & Rogers, 2017)	*FTIR*	OH Stretching Band	5 hours	Does not damage the sample. Fast analysis Quantitative	Short age estimation time Applicable on smooth surfaces..
(Andersson vd., 2017)	*Raman Spectroscopy*	Proteins, Carotenoids, Squalene, and Unsaturated Fatty Acids	1 month	Does not damage the sample. No pre-treatment of the sample is required.	Small sample size.
(Yang & Yoh, 2018)	*Laser-Induced Breakdown Spectroscopy* *Raman Spectroscopy*	CN Bond Amino Acid Saturated Fat	1 month	Trace amounts of samples can be analyzed.	Expensive Short age estimation time
(Muramoto & Sisco, 2015)	*TOF-SIMS*	Position of Palmitic Acid	96 hours	Multiple components can be analyzed simultaneously.	Cannot be used when palmitic acid is degraded. Short age estimation time
(Girod vd., 2016b)	*GC-MS*	Isopropyl Dodecanoate, Cholesterol, Squalene, and Derivatives	36 days	Both volatile and non-volatile components can be analyzed.	May provide incorrect results if storage conditions are unknown.
(Weyermann vd., 2011)	*GC-MS*	Squalene/Cholesterol Ratio	30 days	Sensitive Quantitative analysis	Results are affected by the method of collecting the component from the surface.
(Poletti vd., 2021)	*GC-FID*	Myristic Acid, Palmitic Acid, Stearic Acid, Oleic Acid	30 days	The fatty acid profile of the fingerprint has been characterized.	Small sample size.
(van Helmond vd., 2020)	*UPLC-MS-MS*	Conversion of L-Stereo Isomer to D-Stereo Isomer of Amino Acid	6 months	Enantiomers of various amino acids have been separated.	The impact of environmental factors needs to be examined.
(Oonk vd., 2018)	*LC-MS*	K2C1, K22E, K1C9, K1C10, and DCD Proteins	16 days	Protein mapping of fingerprints has been performed.	The impact of environmental factors needs to be examined.
(Pleik vd., 2018)	*LC-MS*	Ratio Between TG(48:1) and TG(48:0), TG(48:0)-Monoozonide, and TG(48:0)	63 days	Sensitive Quantitative analysis	Damages the sample.
(Frick vd., 2020)	*UPLC-IMS-QToF-MS*	Glyceride Degradation	28 days	Sensitive	The initial amount of ozonoid deposited by the individual on the surface is unknown.
(O'Neill & Lee, 2018)	*MALDI-MS*	Fatty Acid and Triacylglycerol Diffusion	26 days	High resolution Does not damage the sample.	Results vary depending on the surface type.

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A NEW TREND IN FORENSIC TOXICOLOGY: DRIED SAMPLE COLLECTION METHODS

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Chapter 7

A NEW TREND IN FORENSIC TOXICOLOGY: DRIED SAMPLE COLLECTION METHODS

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INTRODUCTION

In addition to the fact that the use of legal/illegal substances and alcohol is a crime, committing a crime under the influence of a substance increases the criminal sanction of the crime due to the indirect effect of the substance, increasing the gravity of the act that causes the crime. In many judicial cases, it is important to determine whether both suspects and victims were under the influence of substances during the judicial incident. In addition, in several administrative procedures, such as drug use, detection of driving under the influence of alcohol and drugs, follow-up of people subject to probation, job and driver's license applications, whether people are under the influence of legal/illegal drugs and alcohol and their history of use can be questioned. Moreover, in various administrative processes, including substance use, detection of driving under the influence of alcohol and drugs, monitoring of individuals on probation, and applications for employment and driver's licenses, inquiries regarding individuals' influence from legal or illegal substances and their usage history may be conducted.

In this regard, biological samples are collected and analyzed. When biological samples are collected by traditional sample collection methods, factors that may compromise the integrity of the sample may occur. For this reason, dried sample collection methods have become a trend in forensic toxicology today due to advantages such as transportation, lower cost, less sample quantity, and less solvent.

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1. BIOLOGICAL FLUIDS AND DRIED SAMPLE COLLECTION METHODS

The identification of legal and illegal chemicals, as well as alcohol, relies on the analysis of the compounds or their metabolites in seized materials and biological samples from individuals suspected of substance usage. Recently, the number of alternative biological fluids in which these substances or their metabolites are detected has increased. These include biological fluids like blood (plasma, serum, or whole blood), urine, hair, oral fluid (saliva), and sweat (Han et al., 2022; Mercolini & Protti, 2016).

These biological samples have advantages and disadvantages over each other. The detection times of legal and illegal substances in biological samples are influenced by the duration of substance usage (acute or chronic), sample collection, analytical method, targeted molecule or metabolite, pH, and matrix concentration (Verstraete, 2004). Detection times for legal/illegal substances are generally found to be the longest in hair, followed by urine, sweat, oral fluid, and blood (Figure 1). The average detection times of substances are in oral fluid (5-48 hours), in blood (1-2 days), and in urine from a few days to 1 month (Langel et al., 2011).

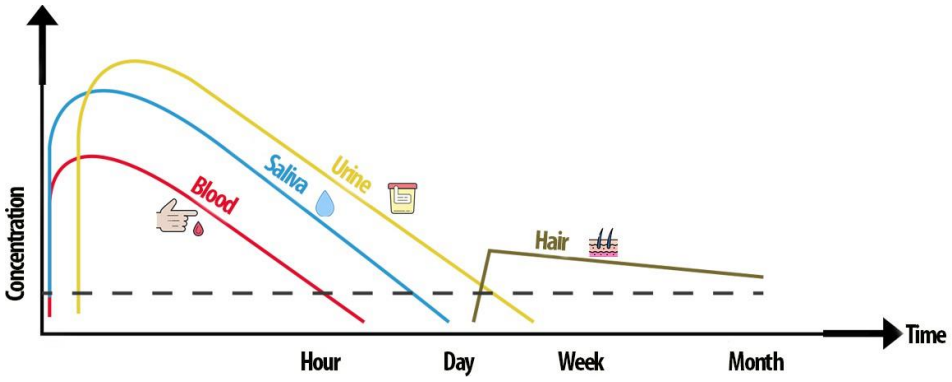

Figure 1: *Detection times of legal/illegal substances in biological samples used in toxicology analysis.*

Nowadays, dried sampling methods have been introduced to overcome the disadvantages of traditional sample collection methods when collecting biological fluids due to less tissue damage, less sampling, lower quantity, lower risk of contamination, and lower transportation costs (Barroso et al., 2022; Han et al., 2022). These sampling methods have attracted great interest as they offer numerous advantages, such as their simplicity, ease, robustness, and reliability. Dried blood samples (Dried Blood Spot, DBS), dried urine samples (Dried Urine Spot, DUS), dried saliva samples (Dried Saliva Spot, DSS), and dried matrix samples (Dried Matrix Spots, DMS) are some of the dried sampling methods used (Grignani et al., 2022; Jacques et al., 2022).

The dried sampling approach entails the collection of a tiny volume (<100 µL) of biological fluid directly onto filter paper, followed by drying, removal of the sample from the paper, extraction, and subsequent analysis (Jacques et al., 2019). Substances at lower quantities can be identified by sensitive instrumental techniques, such as liquid chromatography tandem mass spectrometry (LC-MS/MS) (Gorziza et al., 2020). Dried sample approaches have been employed in preclinical and clinical studies (Detrez et al., 2018), drug development processes (Heussner et al., 2017), elemental analysis (Resano et al., 2018), and toxicological investigations. In toxicological studies, the analysis of substances of abuse (Ribeiro et al., 2019) has been employed in various contexts, such as in workplace drug screening tests (Antunes et al., 2013) in postmortem toxicology (Wilcox et al., 2002), driving under the influence of illegal substances (DRUID) cases (Gorziza et al., 2021), the bloodstains found at the crime scene (Schütz et al., 2002) and doping control in athletes (Han et al., 2022).

1.1. DRIED BLOOD SPOT (DBS)

The use of dried blood samples has been shown to offer numerous advantages over whole blood samples. DBS is preferred as it is minimally invasive/damaging. It is inexpensive to use, easy to reproduce, and automate

over spot testing. The blood sample can be impregnated on filter paper or commercially available cards, stored, and analyzed on the filter paper on demand (Demirev, 2013). This method reduces matrix effects and increases sample lifetime while maintaining analyte sensitivity (Provatas et al., 2019). The use of DBS, especially in problematic populations, such as drug addicts, psychiatric patients, children, and older adults, makes it a comfortable tool (Stove et al., 2012). However, the collection of blood samples must be performed by healthcare personnel (Tey & See, 2021). Due to the use of sensitive instruments, DBS assays can be measured even at analyte concentrations below 1 ng/ml (McClendon-Weary et al., 2020). The DBS approach offers the benefit of yielding a greater number of metabolites compared to analysis utilizing a standard plasma matrix. This is thought to be due to the fact that during the preparation of the plasma, some metabolites are removed along with red and white blood cells during centrifugation (Palmer et al., 2019).

The DBS technique is used in various fields for therapeutic drug monitoring (Verougstraete et al., 2022), newborn screening (Zhan et al., 2022), and disease detection (Wijaya et al., 2021). The DBS technique has been used for doping detection tests in athletes, evaluation of drug-facilitated rape cases, and analysis of postmortem cases in forensic toxicology. Nishio et al. (2023) used the DBS method for the detection of biomarkers on postmortem samples for the analysis of cyanide poisoning (Nishio et al., 2023). Amphetamines, cocaine, opioids including fentanyl and ketamine, along with their metabolites and analogs, were analyzed using specific devices such as LC-MS/MS for the concurrent identification and quantification of illicit drugs and their metabolites in postmortem samples via the DBS method (Odoardi et al., 2014). The analysis of the protein toxins abrin and ricin via LC-MS/MS demonstrated that the DBS approach is applicable for detecting toxins in future forensic toxicological studies (Yishai Aviram et al., 2022).

New psychoactive substances (NPS) refer to newly emerging drugs that are produced by criminals to avoid legal regulations. However, they resemble known illegal substances with their chemical structure and psychoactive effects. The identification and accurate detection of NPS, which are categorized as synthetic cannabinoids, cathinone analogs, phenethylamines, and tryptamines, have an important place in forensic toxicology. Guo et al. (2023) analyzed new-generation narcotics and classical narcotics using dried blood samples (Guo et al., 2023). The analysis of new generation drugs JWH-018, pseudoephedrine, amphetamine, cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene, and norcocaine was performed using the DBS method (de Lima Feltraco Lizot et al., 2019; Thomas et al., 2011).

In forensic toxicology, another important issue in the analysis of biological materials is alcohol analysis. Alcohol abuse is known to have effects on social, behavioral, mental, and physical health. It is necessary to detect abuse to reduce or control the negative effects. Detection of alcohol misuse is important in forensic cases, such as hit-and-run or workplace testing (Sadones et al., 2014). Measuring ethanol to indicate ethanol intake has two major drawbacks: the half-life of ethanol in body fluids is short, and the concentrations of ethanol detected in different parts of the body are different. Therefore, biomarkers other than ethanol itself are needed (Oppolzer et al., 2016). Ethanol biomarkers are direct and indirect biomarkers, and the most important disadvantage of indirect biomarkers is that they only indicate chronic alcohol use. Consequently, the direct ethanol biomarkers Ethyl Glucuronide (EtG), Ethyl Sulfate (EtS), Phosphatidylethanol (PEth), and Fatty Acid Ethyl Ester (FAEE) are predominantly utilized in forensic toxicology (Wurst et al., 2015). PEth has emerged as a prominent direct biomarker for retrospective assessment of excessive alcohol consumption, facilitated by the enzyme Phospholipase D in erythrocyte membranes during alcohol intake

(Andresen-Streichert et al., 2018). PEth can be identified in the bloodstream during a solitary binge of alcohol consumption and persists for 2-3 weeks after an individual ingests a minimum of 50 g of alcohol daily for several weeks and subsequently ceases drinking. The half-life of PEth in blood is four days, and its specificity is notably strong, since it is only produced in erythrocyte cell membranes in the presence of ethanol; furthermore, PEth concentrations correlate positively with alcohol use (Schröck et al., 2017). It has been observed to remain detectable for up to 12 days following a single drinking episode, yielding a blood alcohol content of 1 g/L, and may be identified for up to 3 weeks after repeated intake, remaining stable for years in conserved specimens (Van Uytfanghe et al., 2021).

In a study by Luginbühl et al. (2021), quantitative determination of PEth was performed to monitor alcohol use using DBS (Luginbühl et al., 2021). In the study conducted by Dağlıoğlu et al. (2022), PEth analysis was performed with DBS in samples taken from 50 patients admitted to the hospital as a result of traffic accidents (Daglioglu et al., 2022).

Pesticides are potent and lethal toxic substances used to control and kill pests and can also be used to commit murder due to their accessibility and rapid action (Park et al., 2022). Pesticides were analyzed by Barr et al. (2021) and Lehner et al. (2020) using dried blood samples (Barr et al., 2021; Lehner et al., 2020).

The drawbacks encompass the requirement for healthcare personnel, the potential for contamination, and the hematocrit effect. To mitigate contamination risk, it is advisable to cleanse the initial drop of blood using a sterile gauze swab prior to blood sample collection. In analyses of desiccated blood specimens, fluctuations in hematocrit influence blood viscosity, thereby altering the volume of blood deposited on the filter paper and impacting the size of the resultant spot. Hematocrit alters blood viscosity, resulting in variations in blood diffusion through paper, which adversely impacts the precision and accuracy of quantitative

test outcomes (Jacques et al., 2019; Verougstraete et al., 2022).

1.2. DRIED URINE SPOT (DUS)

Urine is one of the most frequently used biological matrices for detecting wanted substances due to the high concentration of drug/drug metabolites in the urine matrix compared to other matrices (blood, saliva) and its easy collectability. In addition, the detection time of a substance in urine is longer than in blood (Akgür, 2021). However, the stability of urinary metabolites is often poor, and the instability of the sample during transportation and storage poses a challenge to interpreting analytical results. It is time-consuming and requires a complex process, as sample collection needs to be supervised to avoid manipulation (Jacques et al., 2022).

This method's reliability and use have been evaluated by investigations analyzing Dried Urine Spot (DUS) legal and illicit drugs and their metabolites (Gaugler et al., 2022; Stöth et al., 2023). Both legal and illegal substances, along with their metabolites (such as antidepressants, benzodiazepines, cardiovascular medications, neuroleptics, opioids, and stimulants), have been detected using DUS. Furthermore, DUS has been demonstrated as a viable alternative sampling method for extensive drug testing and addiction monitoring programs, including postmortem analysis (Greco et al., 2023; Michely et al., 2017).

Researchers have noted that the DUS method is simple, cost-effective, and offers an alternative for easier sample collection compared to conventional urine analysis methods (Stöth et al., 2023). Therefore, fully automated and robotic methods that analyze dried urine samples to detect legal/illegal substances and their metabolites have been developed and validated (Lauer et al., 2013; Pablo et al., 2020).

The DUS method, like DBS, has the advantages of easy transportation, storage and improved analyte stability. Palmer et al. (2019) compared DBS and DUS samples stored at -20, +4, and +21 degrees celsius with conventional methods (whole blood and urine samples) over a period of 12 months and

found that the metabolites obtained showed higher stability in DBS and DUS samples than conventional methods in all cases examined (Palmer et al., 2019). In addition, the DUS method has an advantage over the DBS method in that legal/illegal substances and their metabolites can be detected in a broader spectrum and higher concentration range and for longer periods of time (Dvořák et al., 2023). Using the DUS technique for detecting alcohol and its metabolites prevents the bacterial degradation seen in classical urine analysis and avoids the erroneous results that can occur in classical urine analysis (Hernández Redondo et al., 2012).

The collecting of urine samples is a more intricate and time-intensive process concerning the chain of custody protocol and oversight of sample collection (Jacques et al., 2022). Thus, an environment should be created in which there are no specially prepared water, soap, or other chemicals, where a supervisor of the same gender can control the sample collection process through a mirror, and where physical and security measures are in place to prevent the urine sample from being altered or foreign substances added to it. In such environments, patients with conditions such as shy bladder syndrome (paruresis) or anuria may not be able to provide a sample. Other limitations of urine sample collection include changes in the concentration of substances due to excessive fluid intake prior to sample collection or dilution by adding different liquids to the sample (Elmongy & Abdel-Rehim, 2016).

1.3. DRIED SALIVA SPOT (DSS)

Although blood and urine are still the most widely used matrices for the detection of illicit substances and alcohol, the use of the oral fluid (saliva) matrix is increasing due to its advantages (Mercolini & Protti, 2016). The DSS (Dried Saliva Spot) method has numerous advantages, such as easy sample collection, low cost, and convenient storage, can be collected by a different gender under direct supervision and without crossing privacy boundaries, does not require invasive intervention, minimizes the risk of manipulation in

sample collection, and ease of transfer (Almeida et al., 2022; Jacques et al., 2019). Another advantage of saliva fluid is that a positive result from the analysis reflects drug use that occurred within the last 24 hours rather than intake that happened days or weeks earlier (Puiu & Bala, 2022; Stevenson et al., 2019).

Saliva, which has a role in digestive function and contains many functional immune substances, is an important fluid for the oral cavity and the whole organism. Saliva is 99% water and 1% inorganic ions, secretory glycoproteins, serum elements, and enzymes. Normal saliva is colorless, transparent, viscous, and tasteless (Diaz-Arnold & Marek, 2002). Salivary fluid can be collected directly into a sampling tube or impregnated onto a pad (Gorziza et al., 2020). The DSS approach offers efficiency and ease in sample preparation. DSS provides a benefit compared to alternative sampling techniques, since it necessitates a minimal sample volume (50 µL) and may effectively mitigate the difficulties associated with plasma or blood analysis. It does not necessitate specialized storage and can be maintained at ambient temperature. Additionally, DSS can be utilized for the screening of medicines, metabolites, and hormones. DSS is a technique that offers advantages for sample collecting, storage, and transportation (Abdel-Rehim & Abdel-Rehim, 2014).

In forensic toxicology, DSS has been used in the analysis of legal/illegal substances and their metabolites, such as phosphatidylethanols (PEth) (Ullah et al., 2017), antiepileptic drugs (Carvalho et al., 2019), methadone and its metabolite EDDP (Ribeiro et al., 2019), cocaine, benzoylecgonine, cocaethylene, amphetamine, MDMA (Jacques et al., 2019) and similar narcotics.

The pH of oral fluid ranges from 5.6 to 7.9, allowing for the detection of weakly basic substances, such as amphetamine, methamphetamine, and cocaine, at larger concentrations than in blood (Bosker & Huestis, 2009; Desrosiers & Huestis, 2019). There are, therefore, advantages to using oral fluid as a matrix in drug testing. Studies have

shown that it has the potential to identify recent substance use in situations such as driving under the influence (Moulahoum et al., 2021; Puiu & Bala, 2022) or workplace drug use monitoring programs (Reinstadler et al., 2019; Risoluti et al., 2020). In addition, using saliva samples also prevents manipulation that can be done in blood and urine analysis. In their study, Gorziza et al. (2020) analyzed amphetamine, methamphetamine, ketamine, benzoylecgonine, and mitragynine using liquid extraction from a paper substrate using the DSS method, followed by liquid chromatography- tandem mass spectrometry (Gorziza et al., 2020). This proved the usefulness and reliability of the DSS method in drug analysis. As a result of a study on the stability of drugs when the DSS method was applied, it was determined that codeine remained stable for 1 day, ecgonine methylester remained stable for

3 days, cocaine, cocaethylene, norcocaine and 6-monoacetylmorphine remained stable for 7 days, morphine remained stable for 14 days, and finally benzoylecgonine remained stable for a total of 136 days throughout the study (Almeida et al., 2022).

The disadvantage of intraoral fluid is that changes can be observed depending on the presence of the substance used, the pH value of the environment, saliva production, contamination of the oral cavity with the smoke of a substances, such as cannabis, polar-apolarity of the substance taken and finally the rate at which it binds to proteins, so all these factors should be taken into account when interpreting (Akgür & Dağlıoğlu, 2018). In addition, in special cases, such as dry mouth, there may sometimes be problems with sample collection (Küme et al., 2016).

CONCLUSION

Due to the problems caused by traditional sampling methods, the use of dried sample collection methods is becoming a trend today. It is understood that the DBS method is the most studied method in the field of forensic sciences, and studies are needed to understand better the relationship between the concentrations of drugs in dried blood samples. Despite this situation, the DBS

method stands out with its advantages over classical methods. Unlike classical methods, the important advantage of the DUS method is that it solves the problems in alcohol analysis by inhibiting bacterial degradation. Studies conducted on DSS have been carried out recently and few compared to the other two dried sampling methods. However, it is noteworthy for its ability to prevent manipulation of blood and urine samples, not requiring specially trained manpower, being particularly advantageous when collecting samples from the elderly and children, not violating privacy boundaries, and providing information on recent substance use. If more studies are conducted on this method, it is clear that the DSS method will greatly benefit the discipline of forensic toxicology.

In Türkiye, it is delivered to forensic laboratories via cargo after the evidence is collected. In this process, disadvantages such as contamination, degradation, high cost, and transportation difficulties are encountered due to the use of classical sampling methods. If dried sampling methods, which make the collection, storage, and analysis of biological matrices, which are important for forensic toxicology, more convenient, easily transportable, have low contamination risk and cost, are made a standard procedure in Türkiye and applied, the problems mentioned above will be prevented and the burden of the justice system will be eased thanks to the contributions of the method in forensic toxicology. In conclusion, it can be seen that dried sampling methods should be integrated more into practice.

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