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Advances in pharmacotoxicological investigation of Sudden Cardiac Death: Literature review and novel perspectives

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ABSTRACT

Pharmacotoxicology is one of the fields that in recent years is growing exponentially, further reaffirming its proven usefulness in the field of forensic sciences. An interesting goal in this field is to identify and quantify the cause of death. This review paper aims to represent a review of the literature in order to evaluate the state of the art regarding the research in the pharmacotoxicology field in cases of Sudden Cardiac Death (SCD), a frequent problem on several occasions, which can be derived from the use of fatal substances or in such concentrations causing sudden death.

In this overview, the most frequently observed biomarkers for SCD were found to be ethanol, illicit drugs, antidepressants, and some common compounds like caffeine and Trenbolone. Concurrently, the most applied instrument configurations is represented by hyphenated liquid (or gas) chromatographic tools coupled with mass spectrometry (LC-MS or GC–MS) in order to reach the high accuracy and confidence levels in the confirmatory analyses, preceded by common sample preparation technique as solid-liquid extraction or liquid-liquid extraction. It was interesting to understand the approaches by which researchers have approached the topic, because on the one hand there are those who have been interested in the comparison between matrices (conventional and not) and on the other hand, who has researched metabolites in order to be able to trace the intake or not of a substance.

In particular, this work want to highlight and evaluate, from a medical-legal point of view, which are the main biomarkers and physiological markers of forensic interest and the methods, and instrumental procedures most frequently used for their evaluation. The paper is organized considering the analytical methods divided by types of drugs/substances.

1. Introduction

With Sudden Cardiac Death (SCD), was indicated an unexpected death in an apparent health patient [1]. Obviously, the term "*sudden*" can indicate an indefinite temporal range [1]. Unexpected deaths from cardiac causes represent a major burden for modern day's medicine and forensic pathologists working on medico-legal cases where the immediate cause of death is prevalently to be found in cardiovascular system. This is true especially in western nations, where Cardiac Disease is easily appreciable as the most common death cause. The general term "*Cardiac Disease*" include vast and heterogeneous pathological conditions that vary from coronary occlusion, valvular problems, cardiomyopathies,

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congenital disease and so on. All these affections are able to provide a florid substrate for the onset of fatal, unpredictable arrhythmias that lead to unexpected death. In many cases, SCD strikes individuals with no prior known history of cardiovascular disease and lacks of macroscopical evidence and poor histopathological founding, lead to a diagnosis by exclusion, which make difficult to establish the exact cause of death in rigorous way, utterly require during jurisdictional authorities cases. From this point of view, all the available complementary investigations may be helpful to further clarify the cause of death in such cases [2]. In recent years, the recognition of the sophisticated interplay between pharmacotoxicology and the occurrence of SCD has been largely investigated [3,4]. In fact, arrhythmogenic and cardiac side effect of pharmaceuticals ad illicit substances is well known, as well known are their implication in precipitating previous cardiac conditions, both acquired and congenital, casting a shadow over medicolegal investigations into SCD cases.

An increase of around 17.5% in drug deaths compared to 2009 was observed in 2019, a worrying figure, arriving about around 500,000 deaths [5]. In those cases, certainly opioids continue to be the leading cause of drug death [5]. In 2021 about 5% of children between 15 and 16 years of age used cannabis, a situation probably intensified by the pandemic period [5]. The worrying fact is the increase in interest in new psychoactive substances (NPSs), characterized by chemical structures similar to those already present, simulating their effects, but avoiding being included in legally controlled substances [6]. The United Nations Office on Drugs and Crime (UNODC) defines these substances as "*substances of abuse, both in pure form and in a preparation, which are not controlled by the Single Convention on Narcotic Drugs of 1961 or the Convention on Psychotropic Substances of 1971 but which may pose a threat to public health*" [7].

Despite these numbers, the problem of sudden deaths is not only due to abuse drugs or to illicit substances, but also to common substances daily consumed/taken. In fact, the exam of literature shows high heterogeneity in types of pharmaceutical, recreational and illicit substances found in SCD cases investigations. An example can be alcoholic cardiomyopathy, which is produced by toxic effects of chronic ethanol abuse [1]. Even taking medication in a not appropriate manner, not following the directions of the prescriber doctor, exceeding the recommended or even maximum therapeutic dose of the drug can cause sudden death, but there are drugs whose effects are not dose-dependent [1]. However, many drugs present even in the most different classes can cause sudden death of the patient. In this field, analytical chemistry can play an key role [8], not only in terms of clinical applications (e.g. therapeutic drug monitoring – TDM) but also in terms of specific procedures for screening/confirmatory purposes in forensic field, helping the pathologist in clarify those cases where no significant pathognomonic signs have been found. As ultraviolet/visible (UV/Vis) and diode array (DAD) detectors have limit of detection (LOD) worse than mass spectrometry (MS), in this review will be find mainly liquid or gas chromatography coupled with mass spectrometry (LC-MS or GC–MS).

It is interesting to note how the interest in the pharmacotoxicological and forensic field coupled with analytical chemistry has increased in the last decades (2010–2024), looking forward to a continuous and increasing interest, as shown in Fig. 1 (data from Scopus database). This is also evident from the research noting an increase in the articles concerning the analysis of post-mortem matrices. For sure, 2024 will increase the number of publications, being only at the middle of this year, continuing on developments on previous years.

This review paper aims to represent an overview in order to evaluate the state of the art regarding the researches in the pharmacotoxicology field in cases of Sudden Cardiac Death (SCD). In particular, this work want to highlight and evaluate from a medical-legal point of view, which are the main markers of forensic interest and the methods/instrumental procedures most frequently used for their evaluation. Specifically, the present review is organized considering the analytical methods divided by the main types of drugs/substances.

2. Ethanol

Ethanol represents a major problem in road accidents [8-12]. This is because ethanol is a psychoactive substance contained in many beverages, which can cause impairment of the state of consciousness, leading to more or less serious short-term and long-term consequences [13]. Furthermore, ethanol possess a well know pro arrhythmogenic effect [14,15]. Thus, it became fundamental to analyse concentration of this compound in SCD cases and after an injury and, usually, the most used matrix for ethanol post-mortem analysis is whole blood, defined in many papers a blood alcohol concentration (BAC) [14,16]. Because in some cases the blood is not always available for most varied reasons, many researchers compared whole blood with new biological matrices. In this case could be right to define these matrices as “unconventional”, because, firstly, they hypothesize the concentration of ethanol in the blood by indirect analysis on another matrix. An example can be vitreous humour, as reported by Savini and colleagues [17]. Their method was fully validated on blood and vitreous humour in gas chromatography-flame ionization detector (GC-FID), obtaining limit of

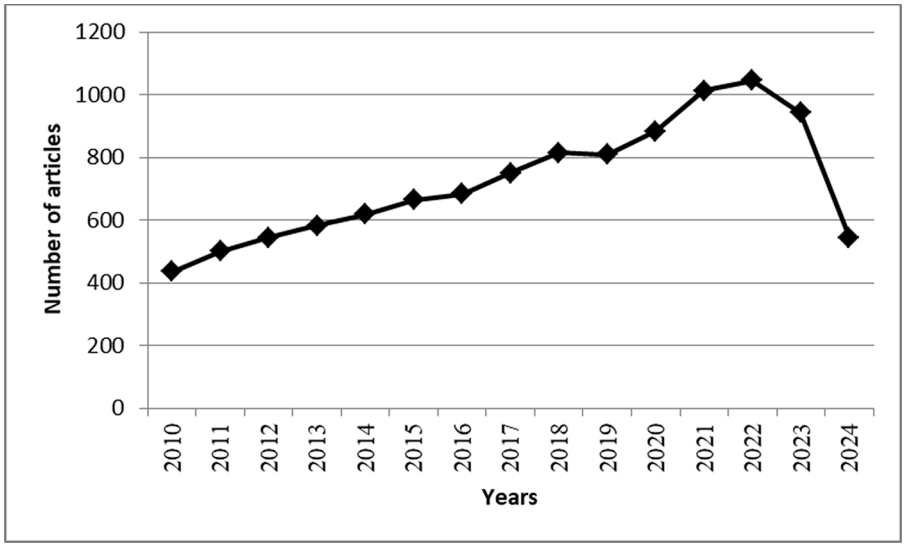

Fig. 1. Graphical interest rate development.

detection (LOD) equal to 0.003 g/L. They confirmed how ethanol's concentration in blood could be influenced by different factors, as temperature, post- mortem interval (PMI), etc. For these reason alcohol in vitreous humour can be higher than in blood [17] especially since the vitreous humour represents a "more closed environment" and less subject to post-mortem changes.

As previously reported, blood is considered as the first choice matrix in the pharmacotoxicology and forensics fields, allowing the possibility of quantifying a wide range of compounds [16]. Ioan et al. in their study considered also urine, because urine is the fastest clearance way for hydrophilic compounds. They found a better correlation between blood and vitreous humour than blood with urine, however this method could be used during toxicological analysis when blood is not present or has been contaminated [13]. In Table 1 are reported some examples of correlation between whole blood concentration of ethanol with others 'unconventional' biological matrices.

Thelander et al. carried out a very extensive research on the correlation between different biological matrices and blood, using a method validated in 1989 by Jones [9,22]. The choice to test so many matrices comes from the ability of ethanol to be absorbed and biodistributed and the concentrations at various fluids/tissues/organs depend on their water content [23]. In addition to the correlation between various biological matrices, another interesting point of view can be also the correlation between ethanol and its main metabolites. Since the interpretation of the concentration of alcohol in the blood is a problem that has always been known, different methods have been validated and studied that can remedy the problem, going to quantify the ethanol ingested ante-mortem and not that formed post-mortem due to microorganisms or synthesis (e.g. blood sugar is converted into ethanol through fermentation) [23-25]. Therefore, in addition to the quantification of ethanol, its main metabolites of phase 1, namely acetaldehyde, ethyl glucuronide and ethyl sulphate, can be investigated [24,25]. Identification of ethanol metabolites proves that ethanol has been ingested, as it has undergone metabolism [26]. On the contrary, the presence of ethanol during autopsy but no metabolites leads to the conclusion that ethanol was probably produced in the body after death [27].

3. Illicit drugs

It is widely known as the drugs of abuse are one of the main reasons for sudden death (intoxication, overdose, etc.). Unfortunately, however, the active ingredient of the substance of abuse, often, represents only a small percentage because both to increase the effects that the gain are often added substances defined as adulterants [26,27]. While pharmacotoxicology is an extremely complex area since there is a need to consider several points like: *i)* the type of analyte, *ii)* the type of a biological matrix considered, *iii)* the stability of the drug itself in the matrix, and *iv)* the type of any metabolites.

On the other hand, this field has the possibility to sample more matrices and, with the right methods, to have a total and as general screening as possible [27]. How previously seen, vitreous humour has large application in this field, but in case of illicit drugs is less used than for ethanol. When possible, bile could be helpful considering that increasing molecular weight of the drug, it will have higher concentration in blood, but bile is used for differing chronic or acute use of heroine [27,28]. Less common is the possibility of using brain, because the interpretation of data results difficult related to the compound biodistribution [26]. Hair analysis was one of the first to be used, thanks to the temporal window that could analyse and long term using or abstinence [11,27]. In fact, it should be highlighted that in the pharmacotoxicological field is possible to move from a time window of minutes for analysis on saliva, to hours (blood), to days (urine), up to months in the case of analysis on keratin matrix (always depending on the specific analyte/metabolite). In Table 2 are reported the main organs/tissues used and for which type of screening.

Cocaine is one of the most relevant substances in SCDs cases. It is an inhibitor of reuptake of dopamine and norepinephrine and is used as local anaesthetic. The indirect sympathomimetic action, contribute to lower the ventricular fibrillation threshold, facilitating proarrhythmic effects, in fact recent cocaine use have been proved to be a precipitating risk factor for Sudden Cardiovascular Death [29]. Cocaine can be

Table 2
Types of matrices correlated to compounds.

Organ/tissue	Type of analysis
Blood/plasma/serum	Preferred for various analytes
Vitreous humour	Ethanol
Bile	Morphine, buprenorphine, BDZ, MDMA
Brain	Morphine, cocaine
Fat	THC
Hair	Many analytes/drugs/drugs of abuse

BDZ= benzodiazepines, MDMA= methylenedioxy-methamphetamine, THC= Δ9 tetrahydrocannabinol

Table 1
Examples of protocols of ethanol analysis.

Matrices	Method	Analyte	Linearity	R2	Ref.
Blood Vitreous humour	GC-FID	Ethanol	0.01–10 g/L	0.9981 ± 0.0025	[17]
Blood	LC–MS/MS	Ethanol Ethyl glucuronide Phosphatidylethanols Fatty acid ethyl esters	0.1–10 ng/mL	> 0.9902	[18,19]
Blood	UPLC–ESI-MS/MS	Ethyl glucuronide Ethyl sulphate	0.06–15 mg/L 0.028–7 mg/L	NR	[20]
Blood Vitreous humour Urine	HS-GC-FID	Ethanol and Acetaldehyde	0.20–1.20 g/kg	NR	[21]
Whole blood Vitreous humour	LC-MS/MS	Ethyl glucuronide Ethyl sulphate	5–10,000 ng/mL	> 0.9993	[22]
Blood Bile Cerebrospinal fluid Vitreous humour Lung fluid Urine Pleural cavity effusion	HS-GC	Ethanol	0.10–4 g/L	> 0.92	[23]

UPLC–ESI-MS/MS= ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry; HS-GC-FID = headspace gas chromatography flame ionization detector; NR= not reported

smoked or snorted. In the first case, it produces anhydroecgonine methyl ester, in the other cases cocaine is subjected to metabolism obtaining ecgonine methyl ester and benzoylecgonine [27]. In Table 3 there are some methods used nowadays for the quantitative analysis of cocaine in biological matrices.

Knuth et al. firstly homogenized 300 mg of brain, and then add isopropanol and acetonitrile (ACN). The sample was centrifuged and then submitted to SPE with dichloromethane, isopropanol and ammonia. Thus, the solvents used are not very green, as required by Green Analytical Chemistry principles [26,35].

Moretti et al. validated a method using LC-MS/MS configuration, comparing blood sample with DBSs. Interesting is the use of DBSs and the perfect according between the results obtained, showing how with only 85 μL of blood can be performed the analysis. They firstly used UAE for 10 min., following by SPE. The eluate obtained from SPE was dried and resuspended in mobile phases. Maybe, it can be considered the possibility of doing just UAE [30].

De Souza Schwarz et al. [31] followed total green protocol, because they used magnetic nanoparticles to extract cocaine, its metabolites and other substances from blood. About 30 mg of nanoparticles were added to 100 μL of blood, vortexed and removed from the solution, which was then added an organic part obtaining phase separation and the latter was directly injected [31]. Nedahl et al. found cocaine in brain and blood in 58 cases, but 44 of them were unrelated with the cause of death. Is important to highlight that cocaine was the principal cause of death in 8 cases reported [32].

Often, an analytical method is not based just on one analyte, but it comprehends more of them. The previously reported methods are based on many illicit drugs, in which can often find methamphetamine (meth) or n-methyl-1-phenyl-propan-2-amine. Meth is a commonly used drug of abuse due to its low cost [36-38]. Researchers think that more than 35 million of people use meth or its analogue [38]. Meth is part of a large group of compounds defined amphetamine, which comprise amphetamine (amph) and meth, both used with recreational/illicit purposes [39]. Meth is the most powerful derivative of amph, thanks to the longer duration of action and better ability to cross the blood-brain barrier. It is, therefore, better known and used in the illegal market [39]. In this category also 3,4-methylenedioxymethamphetamine, commonly defined as MDMA, represent the main monitored analyte [40].

These compounds are rarely involved alone in Sudden Death when taken by the victim, but may have a key role in causing SCDs in case of concomitant drug assumption or in case of pre-existent cardiovascular diseases, which can provide the right substrate for arrhythmogenic mechanism when combined with these type of substances [41]. In Table 4 there are some examples of meth, amph and MDMA extraction and analysis.

It is interesting to mention the work of Klima et al., because is an important example of correlation between common matrices (blood and urine), with new, unconventional, and innovative matrices (teeth) [42]. The extraction protocol based on ultrasonic-assisted extraction is characterized by an extremely green profile. The pulverized tooth has been weighed and, later, has undergone 3 cycles of UAE [42].

Al-Asmari investigated putrefied body and collected firstly only blood, urine, vitreous humour, bile, stomach content, finding how in vitreous humour, for example, meth was detected in 38 of 39 samples tested, showing that vitreous humour could be a good matrix for pharmacotoxicological tests, also in case of death related to the abuse of a mix of illicit drugs [36,43].

Of great concerning, is Cannabis assumption. This because his large popularity among the young make it one of the most detected substances that have been reported in many cases of deaths due to cardiovascular diseases, especially of ischemic nature (e.g. Myocardial Infarction). The leading role of Cannabis and its metabolites in unexpected death cannot be underestimate during forensic investigations [44,45]. Just about THC, it is the most component in cannabis that has psychoactive effects. Its main metabolites are 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THCCOOH) [46]. Fig. 2 reports the main bioactives from Cannabis generally investigated in pharmacotoxicological and medico legal fields.

Cannabis together with alcohol is the most widely used psychoactive

Table 3
Examples of cocaine analysis from different biological matrices.

Analyte	Matrix	Extraction method	Method	Linearity	Ref.
Cocaine	Human brain	SPE	GC–MS	30–1000 ng/g	[26]
Ecgonine methyl ester				105–1000 ng/g	
Benzoylecgonine				93–1000 ng/ g	
Cocaine	Blood and DBSs	UAE + SPE	LC-MS/MS	10–500 ng/mL	[30]
Cocaethylene				10–500 ng/mL	
Ecgonine methyl ester				10–500 ng/mL	
Benzoylecgonine				10–500 ng/mL	
Cocaine	Blood	m-SPE	LC-MS/MS	10–1000 ng/mL	[31]
Benzoylecgonine				10–1000 ng/mL	
Cocaethylene				10–1000 ng/mL	
Ecgonine methyl ester				10–1000 ng/mL	
Cocaine	Brain and blood	SPE	UPLC-TQD-MS	0.005–1 mg/kg	[32, 33]
Benzoylecgonine				0.01–1 mg/kg	
Cocaine	Blood	PP	LC-MS/MS	0.03–0.35 ng/mL	[34]
	Oral fluid	Addition of buffer		0.03–0.25 ng/mL	
	Urine	Addition of buffer		0.02–0.25 ng/mL	

SPE= solid phase extraction; UAE= ultrasound assisted extraction; DBSs= dried blood spots; m-SPE= magnetic- solid phase extraction; UPLC-TQD-MS= ultra-performance liquid chromatography triple quadrupole mass spectrometer; PP= protein precipitation

Table 4
Examples of meth, amph and MDMA extraction and analysis.

Analyte	Matrix	Extraction method	Method	Linearity	Ref.
Amphetamine	Blood and brain	SPE	UPLC-TQD-MS	0.01–0.5 mg/kg	[32, 33]
MDMA	Blood and brain			0.01–1 mg/kg	
Amph	Teeth, blood, urine, hair	UAE	LC-MS/MS	0.13–2400 pg/mg	[42]
MDMA				0.13–2400 pg/mg	
Amph	Blood, vitreous humour, urine, bile, gastric content, liver, kidneys, brain, stomach	SPE	LC-ESI-MS/MS	0.5–1000 ng/mL for fluids	[36]
Meth				0.5–1000 ng/g for tissues	
Meth	Blood, urine, vitreous humour, bile, stomach content	SPE	LC-ESI-MS/MS	1–1000 ng/mL	[43]

LC-ESI-MS/MS= liquid chromatography with electrospray ionization tandem mass spectrometry.

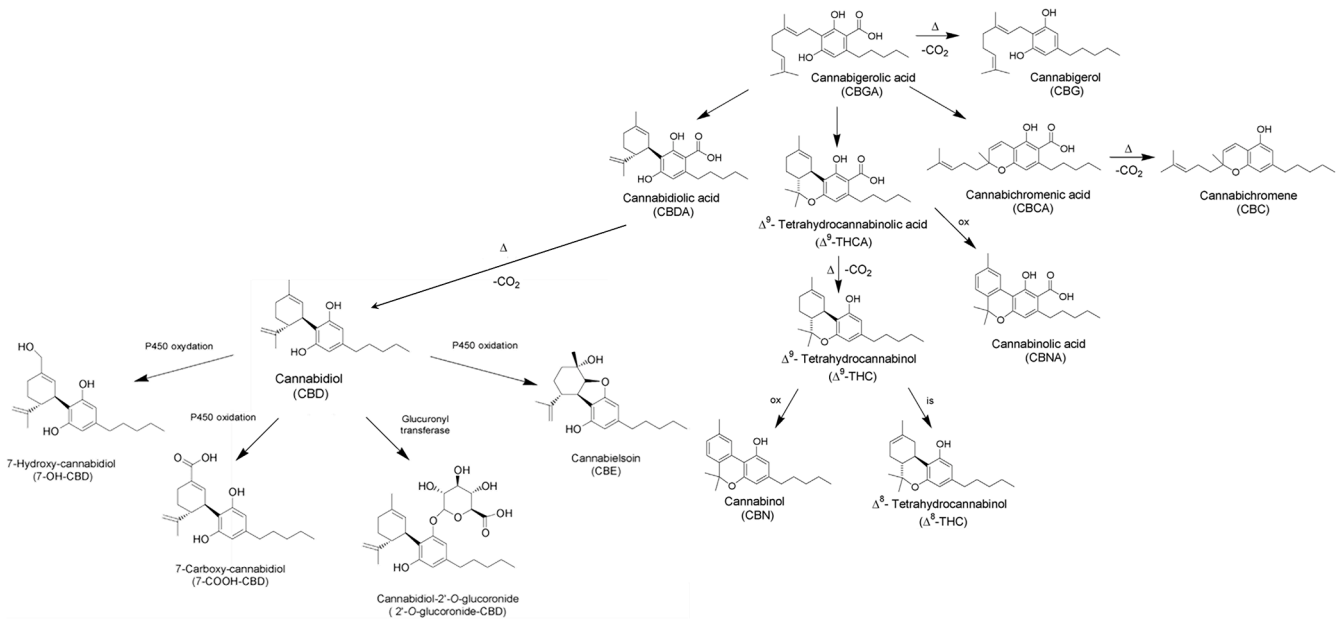

Fig. 2. Main bioactives from Cannabis generally investigated in pharmacotoxicological and medico legal fields.

substance in the world [5]. Over the years the chromatographic technique generally used to detect these analytes was GC–MS, but enzymatic hydrolysis and/or derivatization are time, solvents and cash consuming, and they do not matches with GAC principles [47]. In Table 5 were reported the main configurations (extraction method and instrumental configuration) used for THC and derivatives determination.

The novelty of Cliburn's method is, firstly, the use of LC-MS/MS, in addition the use of a number of important biological matrices, since they have been used blood, urine, bile, liver, lung, kidney, spleen, muscle, brain, and heart. Moreover, the possibility of quantifying so many analytes and not only the major ones like THC, 11-OH-THC, is favourable to the method [47], allowing to obtain a more complete profile.

Lemos et al. in their study started from the consideration that cannabis 'does not kill', which is why it is often not quantified in routine post-mortem screening analyses. They wanted to demonstrate how, on the contrary, the active ingredients were present in most of the analysed matrices. Then, they started by carrying out a screening test with enzyme linked immunosorbent assay (ELISA) to have then quantitative and confirmatory data through GC–MS method [48].

In Hoffman's study, it is interesting to note that THC and THCCOOH often are coupled with ethanol, meth and antidepressant. On the contrary, in 4 analysis THC and THCCOOH were the only drugs detected and these cases was a vehicle-accident, so it is possible that the accident was a direct consequence of the drugs' presence and consumption [49].

Andrews et al. are among the few to use a LLE (liquid-liquid extraction) as an extraction method. It was usually avoided for the type of solvents used and the amount of them. They avoided LC-MS/MS to lower costs. Thanks to continuous research, 2D GC–MS techniques are increasingly developed as it improves detection limits and reduces unwanted background interference without significant financial investments exceeding the cost of standard GC–MS instrumentation [50].

The interesting thing about Saenz's research was that THCCOOH was detected in all liver samples, while THC in 7 of 11 lung. Additionally, lung was found that contain major concentration of THC [51].

Another issue relating to sudden deaths is heroin use. The effect of the latter on central nervous system, produce reduction of respiratory rate and heart rate, thus can lead to both arrhythmic and ischemic disease causing Sudden Cardiac Death. The uniqueness of the heroin is the low half-life (5 min.), which is why it is often necessary to follow its metabolite 6-acetylmorphine, which is the metabolized in morphine [52], as highlighted in Table 6. In 2017, deaths due to heroin and/or synthetic opioids exceeded those caused by opioid analgesics or methadone [53,54].

Al-Amshari reported how it is fundamental in pharmacotoxicological analysis to analyse several tissues as complementary matrices respect to the blood in order to better explore and confirm the use/abuse of a specific illicit compound [55].

Jakobsson et al. validated a complete method on many metabolites of heroin (see Fig. 3) and used urine samples to quantify all compounds, after a screening with an already validated method by Jones [56,57].

The interesting part of Roman's study is the possibility of comparing the real sample with a database of 240 abuse drugs and/or drugs, with the opportunity of quantifying some analytes, but making a generic screening on all the other. This procedure is of fundamental importance in pharmacotoxicology, because, often, the patient's history is not known, so rather than consuming time and solvents this could be an optimal qualifying method [54] before further investigations for confirmatory purposes.

4. Antidepressants

It is of fundamental importance the therapeutic drug monitoring (TDM) and personalized medicine, very popular in recent years, touching the wider medical specializations, having a great impact not only on treatment administration but also even on ascertain eventual professional medical responsibilities during jurisdictional authorities investigations. This is why psychiatry cannot fail to be included in these innovations, especially for all the undesirable effects that psychiatric drugs entail [59]. For this reason in this review, antidepressants are included. Many times, with the aim of reducing dosages, medical doctors use combined therapy, but this represents a problem for sudden cardiac death [60,61]. Antidepressants, along with benzodiazepines and others medications commonly used in psychiatry, may have, especially when combined in polypharmacological therapies, notable effect on cardiac conduction system, leading in such manner to sudden death [11], especially if they possess QT-prolonging action [62]. In Table 7 were reported procedures used for antidepressants determination in biological fluids.

It is interesting how Sempio et al. validated a method for quantifying 88 drugs (BDZ, antidepressants and antipsychotics) using LC-MS/MS with a Hypersil Gold column with gradient elution and flow rate of 0.2 mL/min in only 20 min using as internal standard a drug that cannot

Table 5
Examples of THC and derivatives extraction and analysis.

Analyte	Matrices	Extraction method	Linearity	Method	Ref.
8β-diOH-THC THCCOOH-g THCVCOOH THC-g 8β-OH-THC 11-OH-THC THCCOOH THCV CBD CBG CBN THC	Blood, urine, bile, liver, lung, kidney, spleen, muscle, brain, heart	SPE	1–100 ng/mL 1–250 ng/mL 1–100 ng/mL 0.25–50 ng/mL 1–100 ng/mL 0.5–250 ng/mL 0.5–250 ng/mL 1–100 ng/mL 0.5–250 ng/mL 0.5–250 ng/mL 0.5–100 ng/mL 0.5–250 ng/mL	LC-MS/MS	[47]
THC THCCOOH 11-OH-THC	Blood and urine		1-? ng-mL 11-? ng-mL 5-? ng-mL	GC–MS	[48]
THC THCCOOH	Blood	PP+SPE	1–200 ng/mL 1–200 ng/mL	GC–MS	[49]
THC CBD CBN 11-OH-THC THCCOOH	Blood	LLE	0.25–50 ng/mL 0.5–50 ng/mL 0.25–50 ng/mL 0.25–50 ng/mL 0.5–50 ng/mL	GC–MS	[50]
THC 11-OH-THC THCCOOH	Blood, urine, vitreous humour, liver, lung, kidney, spleen, muscle, brain, heart and bile	PP/hydrolysis+SPE	0.78–100 ng/mL	UPLC-MS/MS	[51]

8β-diOH-THC= 8-Beta-diHydroxy-THC; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; THCVCOOH: 11-nor-9-carboxy-THC; THC-g: THC-glucuronide; 8β-OH: 8-Beta-Hydroxy-THC; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THCV: Δ9-tetrahydrocannabivarin; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THC: Δ9-tetrahydrocannabinol; LLE= liquid-liquid extraction

be prescribed in Italy (Halazepam) [63]. The great novelty of these methods is that, finally, they used an extraction method that fully matches with GAC principles (dilute and shoot).

Øiestad et al. validated their method on several matrices, having regard to the possibility of the absence of blood from bleeding or rotting [64]. They screened their samples with UPLC-MS/MS, but later validated on UHPLC-QTOF-MS with a reversed phase C18 column and a high pH mobile phase. Amundsen validated the first method used in 2013 [67].

Finally in 2022, thanks to a new extraction techniques, has been validated a method in HPLC-PDA Locatelli et al. have used a brand new extraction method (patented in 2014 by Kabir and Furton [68]) defined

Table 6
Examples of analytes extraction and analysis for heroin detection.

Analyte	Matrices	Extraction method	Linearity	Method	Ref.
6-MAM 6-AC Morphine Codeine	Blood, vitreous humour, urine, stomach contents, bile	SPE	0.001–1.00 mg/L	LC-MS/MS	[55]
M3G M6G NMOR MOR C6G NCOD COD 6-MAM EMOR	Urine		0.2–100 μg/mL 0.2–100 μg/mL 0.05–5 μg/mL 0.025–50 μg/mL 0.2–100 μg/mL 0.05–5 μg/mL 0.025–25 μg/mL 0.01–10 μg/mL 0.02–25 μg/mL	UPLC-MS/MS	[56]
MOR 6-MAM	Blood	PP + SPE	0.005–2.0 mg/L	GC–MS	[57]
Ethylmorphine	Blood	PP	–	LC-TOF-MS	[58]

6-MAM= 6-monoacetylmorphine; 6-AC= 6-acetylcodeine; M3G= morphine-3β-D-glucuronide; M6G= morphine-6β-D-glucuronide; NMOR= normorphine; MOR= morphine; C6G= codeine-6β-D-glucuronide; NCOD= norcodeine; COD= codeine; EMOR= ethylmorphine

Fabric Phase Sorptive Extraction (FPSE). FPSE method allow avoiding filtration, protein precipitation or longer extraction method, because through the optimization of sample volume, extraction time, back extraction time, solvent and volume it obtains directly the sample ready to inject [8]. Obviously, the parameters to optimize were tested through one variable at time (OVAT) design. This method matches extraction/preconcentration with Green Analytical Chemistry (GAC) and Green Sample Preparation (GSP) principles.

Campelo et al. validated an interesting method to quantify 20 antidepressant, taking advantages from QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe). They optimized several variables like solvent, agitation mode, sorbent clean up, type of partitioning salts. With the aim of fully validated parameters, they used a 24 experimental design, in random order to evaluate the chromatographic response [65].

5. Common substances

Very unexpected is the research of caffeine in post-mortem toxicological analysis. This because a recent study identified an association between antiepileptic/caffeine and pathogenic drugs, playing an essential role as triggers of SCD in genetically predisposed young people [69]. In Sweden, caffeine occupies 19th place in substances found in the body during autopsies. This is because, nowadays, the power of this is underestimated and it is extremely easy to find energy drinks and/or tablets containing caffeine or its derivatives on the market [70].

In a case report made by Szeremeta, toxicological tests of the corpse showed a caffeine concentration equal to 92.0 μg/mL in blood, and no other substance of abuse, drugs or alcohol has been found [71].

Other cases report from 1999 to 2009 described 8 different intoxication of caffeine. The analysis were done on GC–MS, through a liquid-liquid extraction [72]. Examples of substances that can cause Sudden Death could be synthetic anabolic androgenic steroids (AAS). Thanks to

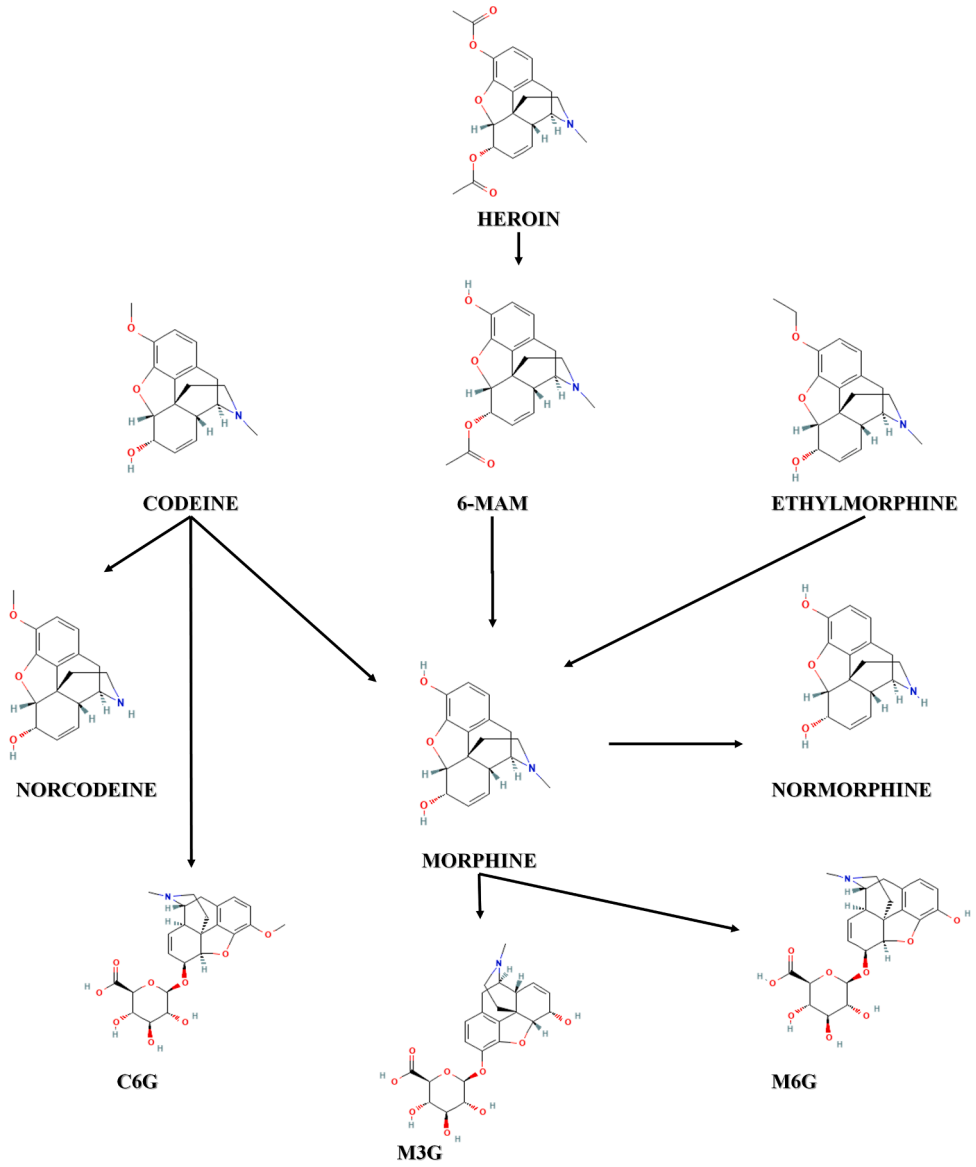

Fig. 3. Schematic representation of the heroin metabolism.

their properties, athletes for doping aim use them and for this reason, World Anti-Doping Agency has prohibited them [73]. Gheddar reported an example of quantifying Trenbolone in different matrices in a post-mortem case [74].

They used different biological fluids (as blood, bile, vitreous humour) and hair to matches the results. The first screening was made on ethanol with headspace gas chromatography with flame ionization detection (HS-GC-FID) in blood, meanwhile liquid chromatography for others substances. Specifically, the group used an UPLC-Q-TOF-MS with a quantification method of only Trenbolone and Testosterone. Through a gradient with acetate buffer and acetonitrile, they were able to resolve the two compounds, finding Trenbolone in all fluids used and hair. With a linearity from 1 up to 500 ng/mL, they find good correlation between cardiac and femoral blood, demonstrating that was not redistribution. Instead, in bile and cardiac blood they highlight the presence of Trenbolone and its metabolite, confirming the assumption of the drug [74].

6. Greenness of procedures

Nowadays, one of the most important aspect is to follow GC, GAC and GSP principles, which are based on miniaturization in every field, from sample to solvents used to waste. Firstly, often to ensure method sensibility a detector as mass is used and it could not total matches with these principles because it uses a lot of energy and it need to be used from qualified personnel [35].

For example, Moretti and colleagues compared DBS with blood, using 85 μL of blood on a card and kept in a dark room. For sure, using DBS is '*greener*' than using blood, but it could be greener if they used as sample preparation only UAE and not SPE, because there is the necessity of more samples and solvent used [30].

Interesting is De Souza Schwarz's study in which they used unmodified commercial Fe3O4 nanoparticles as a sorbent for dispersive magnetic solid-phase extraction. They used 100 μL of blood, added to 30 mg of nanoparticles [31]. Green and with a big number of analytes is the study conducted by Tana et al. in which they quantified 77 NPS, 24 drugs and 18 metabolites using protein precipitation (one step) and the addiction of a buffer, that is subjected to dryness and then suspended in mobile phase [34]. Very interesting is the method provided by Klima and her team, because they correlated various matrices using as extraction method only UAE, and obtaining very low LOD [42]. At the meantime, it could be used FPSE, as Locatelli and colleagues did. This type of extraction method matches small amount of samples (500 μL of

Table 7
Procedures used for antidepressants determination in biological fluids.

Analytes	Matrices	Extraction method	Linearity	Method	Ref.
88 drugs (BDZ, antidepressants, antipsychotics)	Blood	Dilute and shoot		LC-MS/MS	[63]
Amitriptyline	Blood, urine, pericardial fluid, psoas muscle, lateral vastus muscle, vitreous humour	LLE	0.0055–0.055 mg/L	UHPLC-QTOF-MS	[64]
Citalopram			0.0032–0.032 mg/L		
Mianserin			0.0026–0.026 mg/L		
Mirtazapine			0.0013–0.013 mg/L		
Nortriptyline			0.0026–0.026 mg/L		
Paroxetine			0.0017–0.016 mg/L		
Sertraline			0.0076–0.015 mg/L		
Trimipramine			0.0029–0.029 mg/L		
Venlafaxine			0.0055–0.055 mg/L		
Venlafaxine	Blood and cerebrospinal fluid	FPSE	0.2–20 μg/mL	HPLC-PDA	[8]
Citalopram			0.1–20 μg/mL		
Paroxetine			0.1–20 μg/mL		
Fluoxetine			0.1–20 μg/mL		
Sertraline			0.1–20 μg/mL		
Amitriptyline			0.1–20 μg/mL		
Clomipramine			0.1–20 μg/mL		
20 antidepressants	Blood	QuEChERS	10–1000 ng/mL	LC-MS/MS	[65]
16 antidepressants 7 antipsychotics	Blood	QuEChERS	1–500 ng/mL	LC-MS/MS	[66]

UHPLC-QTOF-MS = ultra-high performance liquid chromatography quadrupole-time-of-flight mass spectrometry;

blood and liquor), simple and quick extraction procedures, that due less preparative error, that is widely demonstrated that this part of the analytical procedure is the major subjected to errors [8], as explain in Fig. 4.

It is also highlighted how the FPSE technique can also be used for the determination of volatile compounds [75], paving the way for its future applications in the medico-legal field related to the research of specific biomarkers, drugs, or metabolites.

Important is also the applicability of the procedures, because many times some laboratories could not have some instrument or follow some protocols. Thus, a simple sample treatment and the use of low microliters of sample could help. In fact, since in some deaths due to some conditions (putrefaction, permanence in uncommon environments, uncertainty about the causes of death or timing) it is impossible to sample common matrices such as blood, studies comparing a conventional matrix with a minus matrix are of fundamental importance, so that exact cause of death and quantification of both lawful and non-lawful substances can be established in one way or another. In laboratories dealing with this field, the possibility of using nanomaterials based on biosensors is increasingly being considered. Certainly interesting and innovative, but more difficult to find, classic example is the search for alcohol dehydrogenase, inside which there is zinc in order to bind it to gold nanoparticles covered with thioctic acid [76]. This could be an optimal green approach, the only problem is the developing of a protocol and materials availability.

7. Future perspective

The analytical results following SDC are of fundamental importance when related to the specific investigated case (autopsy findings, type of death, age, coverage of pathologies, circumstantial data) and only in this case can have an effective response. However, the interpretation of the data turns out to be a narrow obstacle, as post-mortem redistribution of drugs (abuse or not) can make the analysis inefficient, thus reducing the credibility of the evidence. For example, it is widely known that co-administration of alcohol and cocaine increases the half-life of the latter, whereas heroin, when taken together with alcohol, suffers increased toxicity [77]. For this reason, the pharmacotoxicological analytical field requires selective, specific and rapid-response methods, so that a quantification can be given as soon as possible. Therefore, we will try to prefer, in the future, simple and quick extraction techniques, such as *dilute-and-shoot* or protein precipitation. When these methods

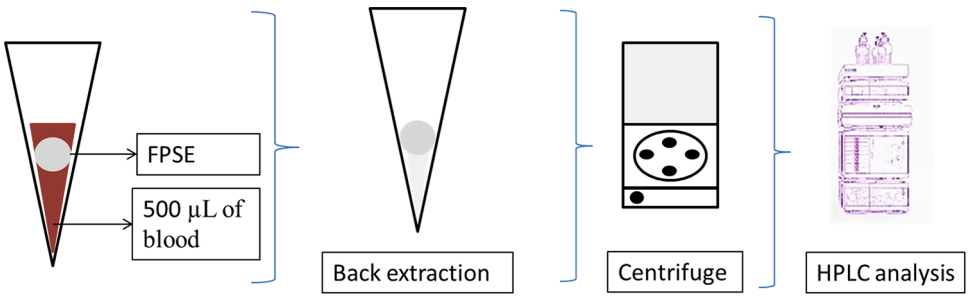

Fig. 4. Schematic representation of FPSE procedure.

are not appropriate (for the type of matrix), green methods such as FPSE or the use of nanoparticles or micro extraction will be preferred. About this in Table 8 are reported advantages and disadvantages of most used technique reported in this review.

Many review/ article are based on researching from post mortem matrices, but often they are focused on one type of matrix or one analyte (or one class) (different from this type of research) [78-80]. All of them are agree about one of the main goal of extraction technique, which can be summarized in 'miniaturized' for samples and solvents used, but also for energy consumption. Maybe, the major problem could be energy consumption due to instrument used. For these reasons, new materials/instrument/devices will be used and developed based on this idea and with the aim of reducing environmental pollution. The best goal will be a total minimisation of the abovementioned parameters.

Additionally, this field is constantly growing, as every day new psychoactive compounds are detected. For these reasons, the search for methods for the effective quantification of substances (abuse and not) requires a continuous development. The absence of data about a new compound could ruin an entire forensic investigation with the penal consequences it entails.

Another challenges that every report remember is the necessity of accurate, precise and strong method, that matches both capacity and instrument of most laboratories, avoiding errors, because, often, different laboratories can give different results.

8. Conclusion

Investigating the causes behind Sudden Cardiac Death may be quite a challenge for forensic pathologist, especially when the autopsy is "silent". In such cases, become essential a multidisciplinary approach and pharmacotoxicological investigations could prove to be crucial for the case resolution. So is of outmost importance to relay on modern, reliable and precise methods, especially with the continuous introduction of new substances that cannot be detected with old techniques.

In the present work, was highlighted that despite scientific progress, most of the developed and widely used methods in the pharmacotoxicology and forensics fields consider the use of LLE or SPE as the sample preparation phase. These procedures are too consuming in terms of time, reagents and solvents and matrix, and they do not match with Green Chemistry and Green Sample Preparation principles.

As seen, the most used instruments are GC or LC coupled with mass spectrometry, which is more expensive as a detector than a PDA. It is clear how LC-MS configurations allow obtaining levels of selectivity and sensitivity that satisfy and respond to the needs in the pharmacotoxicology and forensic fields. In these cases, the problem of the limit of detection is not so easy to overcome, but at the same time, is hopefully that in the future methods can be considered the use of a more innovative and less expensive extraction techniques, to avoid the MS drawbacks regarding the GAC principles.

Table 8
Advantages and disadvantages of the techniques reported in this work.

Technique	Advantages	Disadvantages
Dilute and shoot	✓ Rapid ✓ Economic ✓ Optimal reovery ✓ Cheap	• If analyte i slow concentrated, it needs a method with low LOD
LLE	✓ High quality ✓ Low risk process ✓ Easy ✓ Good recovery	• Time consuming • Solvent consuming • High power consuming • Types of solvents used
SPE	✓ High quality ✓ Low risk process ✓ Easy	• Time consuming • Solvent consuming • Types of solvent used • High power consuming
FPSE	✓ Rapid ✓ Low solvent consuming ✓ Good on different types of matrices ✓ Possibilty of changing the surface based on chemical properties of analytes ✓ Safe	• Necessity to have devices • It needs optimization of extraction
QuEChERS	✓ Quick ✓ Easy ✓ Cheap ✓ Effective ✓ Rugged ✓ Safe	• Optimization of extraction solvent • It needs kit QuEChERS
PP	✓ Rapid ✓ Simple ✓ Economic ✓ Low solvent consuming ✓ Safe ✓ Cheap	• If analyte is linked to protein it may precipitate
UAE	✓ Rapid ✓ Low solvent consuming ✓ Green ✓ Easy	• Necessity to have ultrasound instrument • It needs attention to temperature
Gold nanoparticles	✓ Sensitive ✓ Rapid ✓ Safe ✓ Efficient extraction	• Necessity to have specific material • Longer procedure than others (synthesis + extraction)

An interesting approach is to continue the research for "innovative" and unconventional matrices that can be used in routine analysis when the classic matrix is absent, which is very important in forensic field, where the canonical matrix not always is available. The necessity in such cases to find alternative matrix that can be optimize for application in unconventional situations, represents an additional challenge in the innovation of forensic pharmacotoxicology. As well as interesting turns out to be the search for metabolites of the drug to follow, if this is unstable or rapidly metabolized, especially in cases where you are not aware of the estimated time of death.

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CRediT authorship contribution statement

Antonio Maria Catena: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Marcello Locatelli:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Miryam Perrucci:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Vincenzo De Laurenzi:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Imran Ali:** Conceptualization, Data curation, Methodology, Visualization, Writing – review & editing, Writing – original draft. **Luigi Miccolis:** Conceptualization, Data curation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Andrea Mazzatenta:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Fabio Savini:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Cristian D'Ovidio:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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