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A pilot study on post-mortem determination of drug abuse on dental tissues

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ABSTRACT

Background: Post-mortem toxicology constantly deals with the research of reliable alternative matrices to be applied in case of highly damaged corpses (such us carbonized, skeletonized, human remains, etc.). Teeth represent a promising alternative matrix since dental tissues are endowed by different features, resistance and stability after death.
Scope: Since scant literature reported on the pharmacokinetics and mechanism of incorporation of xenobiotics into dental tissues, this pilot research aims to investigate whether in the pulp can be detected the same substances found in blood in drug related death cases. Secondly, the study is addressed to disclose the possible deposit of drugs in dental hard tissues (dentine and/or enamel), thus contributing to reconstruct the drug abuse history (timing, e.g.).
Materials and methods: The study experimented with a novel method to separately analyse dental enamel, dentin, and pulp, applied to 10 teeth collected during autopsies of drug-related deaths along with blood and hair samples for classic toxicological analyses. Each tooth was prepared by "pulverization technique" and then analysed by gas chromatography paired with mass spectrometry (GC-MS) and ultra high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC/HR-MS) for searching cocaine, opiates, and metabolites. The results were then compared with those obtained from blood and hair samples.
Results: Preliminary results demonstrated that teeth differ from any other classic matrix (blood and hairs) since the qualitative correspondence of the detected substances between pulp and blood as well as dental hard tissues and hair suggests that they can be useful in post-mortem evaluation as a unique matrix for both acute and chronic assumptions of drugs. The mechanism of accumulation of substances in mineralized dental tissues emerged the most significant result, being influenced by the type of molecule and the method of assumption. The main limitation of this study is the limited availability of the sample and the absence of anamnestic information of the time, rates and method of drug assumption during life. Further research is necessary to systematically investigate the distribution of different substances within the different tissues of the tooth.

1. Introduction

Post-mortem (PM) toxicological analysis is often complicated by numerous artefacts caused by cadaveric alterations. Some drugs (e.g. cocaine, heroin, methadone, benzodiazepines) are more susceptible to cellular autolysis, blood coagulation, and hypostasis [1–6]. Blood, fluid, and tissues' concentration levels are also influenced by physical or chemical characteristics of the assumed substance (acid/basic properties, lipid solubility, protein bonds' formation, volatile molecules, etc.) [7–9]. Furthermore, bacterial activity due to progressive PM processes can increase or reduce the concentration levels of different substances in different anatomical districts [10]. Bacteria themselves can degrade drugs and their metabolites as well as biological tissues ("post-mortem bioconversion"), with progressive accumulation of *interferents* which can

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“mask” concentration levels [7,9–12]. The PM redistribution of substances from "reservoir" organs towards the blood and surrounding tissues is one of the most studied PM phenomenon [9]. The redistribution patterns are mainly influenced by the characteristics of the substances (for example, lipophilic and alkaline), and the anatomical site of the toxicological sampling, since central matrices present a greater redistribution due to their proximity to the thoraco-abdominal organs [2]. A further issue in PM toxicology is determining whether the quantity of the taken substance could cause or contribute to cause the death. A subject chronically exposed to certain substances, in particular morphine and cocaine, may have developed a form of tolerance during life and then it is necessary to progressively increase the dosage to obtain the expected therapeutic or narcotic effect [13]. The toxic or lethal dosage of each exogenous substance must be investigated and interpreted case-by-case deepening the state of health and the specific intake habits of the subject while alive. The choice of PM matrices which could offer a window of detectability of weeks/months/years is therefore pivotal to reconstruct the history of a chronic or occasional use or abuse of substances [14,15]. In some particular cases, the toxicological investigations and the choice of reliable matrices are affected by the complete, or almost complete, destruction of the conventional cadaveric matrices [blood, fluids, urine, or organs up to hair]. The availability of these matrices presents strong limitations due to the preservation of the PM body, the time elapsed since death (post-mortem interval - PMI) and the circumstances of death. Very few biological matrices resist unchanged to advanced putrefactive phenomena, high temperatures, extremely humid or extremely dry environments, the skeletonization of bodies, exhumation or the action of macrofauna in an open environment [16–18].

In this scenario, teeth represent a potential alternative matrix for PM forensic toxicology applicable for the diagnosis of both acute intoxication and the history of chronic substance abuse. Teeth are highly resistant to environmental agents even in extreme conditions (heat, humidity, dehydration, pressure, etc.) and stable for late PMIs due to their strategic anatomical position inside the oral cavity, and protected by the perioral tissues [19–23]. Moreover, the high mineralization of the external tissues of dental crowns renders teeth poorly susceptible to post-mortal changes and environmental agents, and preserves the internal dentin and pulp [24–26].

Due to the different development and constitution of dental tissues, xenobiotics could accumulate inside the tooth with different mechanisms depending on the dental site. In the enamel (external and mineralized tissue), as well as in the primary dentin (deposited from the early stage of tooth formation until its complete maturation) they can accumulate either during the intraosseous mineralization of the crown or by contamination from the oral cavity. For the secondary dentin (deposited in a centripetal manner by the maturation of the tooth throughout the subject’s life), the accumulation mechanism of substances can occur from the blood flow or external contamination. The dental pulp is vascularized by peripheral blood and the presence and concentration of substances could be compared to hematic ones. Previous literature demonstrated that many substances of abuse and drugs can be detected in teeth [27–33]. Cattaneo et al. [30], Klima et al. [31], Ottaviani et al. [33], and Cippitelli et al. [23] analysed teeth from cases of burnt, putrefied, skeletonized remains, and exhumed bodies found in conditions strongly suggestive of a drug-related death and results showed that different drugs could still be identified in dental pulps, as well as in dental hard tissues in cases of history of abuse. The toxicological window of detectability, in particular for amphetamines, 3, 4-Methylenedioxymethamphetamine (MDMA), methadone and 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), even seems to overlap with that of hairs, exceeding the diagnostic possibilities of blood samples in the context of chronic intake [30]. These preliminary scientific findings confirmed that dental tissues represent a reliable source of toxicological information for both acute intoxication and history of abuse.

This pilot study aims to develop a method to analyse different dental tissues according to an innovative technique for separating enamel, dentin, and pulp. Secondly, the research is addressed to disclose the mechanism and site of absorption of xenobiotics in dental matrices, to verify whether the substances circulating in blood can be detected in dental pulp in cases of death related to overdose/acute intoxication and the deposits in the mineralised tissues of the tooth, dentin and/or enamel, can support the diagnosis of a chronic substance abuse. The last objective is to verify the reliability of two methods, one developed by Ottaviani et al. [33] on the entire tooth and one developed by Buratti et al. [34] on alternative keratinized matrices (nails), applied separately on different dental tissues (enamel, dentin, and pulp).

2. Materials and methods

2.1. Reagents and materials for toxicological analyses

Proadifen (SKF) internal standard (IS) for the analysis in gas chromatography (GC-MS) and ultra high performance liquid chromatography with high-resolution mass spectrometry (UHPLC/HR-MS), and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) were purchased from Sigma.

Methanol (MeOH), water, hydrochloric acid (HCl), dichloromethane, 2-propanol, ammonium hydroxide of reagent grade for the solid phase extraction (SPE), methanol (MeOH) for UHPLC, Formic Acid (FA) for UHPLC and ultrapure water (H2O) for UHPLC were obtained from Carlo Erba; SPE Columns and Well Plates - Isolute HCX cartridges (130 ng/10 ml) from Biotage.

All reagents were of analytical grade and stored according to the manufacturer’s instructions.

2.2. Sample collection

The experimental study was conducted on real cases of drug related death and PM dental specimens have been extracted during autopsy in two different Italian Forensic Centres (Personal Identification and Forensic Morphology Laboratory of the University of Florence and the Forensic Medicine Laboratory of the University of Macerata).

The inclusion criteria were:

- drug-related deaths, regardless of different kind of substance of use/abuse or deaths due to different cause, but of subjects with a history of drug use/abuse who underwent during life to classic toxicological investigations on blood and hair samples.
- healthy teeth extracted intact (no cavities, conservative or endodontic treatments, etc…); absence of severe wear.

The exclusion criteria were:

- cases that did not meet one or more inclusion criteria.

A case of a non-addicted subject was included in the study as a negative control of analysis and assumption.

Where possible, the following anamnestic data of the deceased subjects were collected: sex, age, previous history of drug addiction or detoxification, clinical history regarding the specific pharmacological therapies followed, previous pathologies. Sex and age of the dead subjects were not currently considered significant factors for the inclusion/exclusion of the sample.

For the selection of the post-mortem tooth to be extracted, all available samples were taken into consideration without selecting the type of dental element.

After the pulp extraction and the hard dental tissues separation, each sample was assigned an alpha-numeric code consisting of the name/number of the autoptic case (e.g. ILE1) and a letter corresponding to the specific dental tissue (in particular, "P" for pulps, "S" for the enamel, "DCC" for the central coronal dentin, “DCP” for peripheral coronal

dentin, and “DR” for radicular dentin).

2.3. Dental tissues separation

Each dental specimen extracted during autopsy was washed with saline solution and decontaminated in sodium hypochlorite for 5–10 minutes. Each tooth was assigned with an alpha-numeric code to anonymize the deceased subject’s data and prepared by the same forensic odontologist at the same dental unit, using diamond and/or multi-blade burs mounted on a turbine. The separation of the tissues took place above a switched on amalgam aspirator covered with filter paper, without cooling water.

Teeth were divided into two longitudinal halves to access the pulp at first. A 360° “guide” groove was made on the external surface of the tooth with a diamond bur. The groove varied in thickness depending on the type of tooth. The tooth was not completely separated with the bur to preserve the dental pulp but the two dental halves were separated with a hammer blow on an extraction lever positioned within the area of least resistance obtained with the "guide" groove. Then the pulp was extracted from the chamber and roots with an excavator, surgical tweezers and nerve pullers. Once extracted, it was inserted into a test tube on which the alpha-numeric code of the reference case was written followed by the indication "P" (“pulp”).

Then, we proceeded with the preparation of the mineralized tissues in enamel, peripheral coronal dentin (considering the inclusion of only the already mature and fully formed primary dentin [35–38]), central coronal dentin (considering the inclusion of the secondary dentin deposited during the subject’s life [35–38]), root dentin.

Starting from the coronal tissues, using a multi-bladed bur on a completely dry tooth:

- the enamel was separated from the peripheral coronal dentin by pulverization. The enamel layer was easily recognized from the underlying dentin given the different colour of these tissues;
- the central (secondary) coronal dentin was separated from the peripheral (primary) coronal dentin by pulverizing an internal layer of approximately 1.5 mm of coronal thickness and 1 mm of cervical thickness, by approximation using millimetre probes [39,40];
- the residual peripheral (primary) coronal dentin was separated from the roots by pulverization;
- and then the roots were pulverized with a hammer. Considering that the cement could not be contaminated either from the inside or from the outside, the entire root was considered as a single sample for the analysis of dentine alone.

Once the different tissues had been pulverized, each sample of powders was inserted into a test tube on which the alpha-numeric code of the reference case was reported followed by the indication "S", or "DCC", or “DCP”, or “DR” respectively for the enamel, central coronal dentin, peripheral coronal dentin, and radicular dentin.

Each dental tissue and conventional matrices (blood and hair) underwent the extraction for opioids, methadone, cocaine, and their metabolites and then were analysed with the same procedures (UHPLC/HR-MS and CG-MS) to compare results.

2.4. Toxicological analyses on different dental tissues

2.4.1. Opioids and methadone extraction and analysis

For opioids, methadone, and their metabolites an already validated method on different alternative matrices (nails) was adapted for dental tissues [34]. Each pulp/hard tissue sample was supplemented with 2 ml of 0.1 N HCl solution. 20 ng of IS (SKF) was added. Samples were incubated for 72 hours at 55°C. After incubation, they were centrifuged at 14,000 rpm in an ultracentrifuge and the supernatant was collected and evaporated. Subsequently, they were resuspended with 50 µl of mobile phase B (MeOH + 1 % FA) for chromatographic injection.

The Thermo Scientific Dionex Ultimate chromatographic system 3000 (UHPLC) was used for the analysis coupled with the Thermo Exactive Plus Orbitrap mass spectrometer (HR-MS). The conditions applied for the chromatographic analysis were as follows: the column used was Kinetex Biphenyl 2.6 µm (50 ×2.1 mm) from Phenomenex; the column flow was set to 0.4 ml/min. The phases used were the following: phase A: H2O + 0.1 % formic acid; phase B: MeOH + 0.1 % FA. The column temperature was set to 25°C. The elution gradient was: 98 % phase A 0–0.5 min, up to 100 % phase A in 10 minutes; isocratic 100 % phase A for 2 minutes, 98 % phase A from 12 to 15 minutes. The exact mass (EM), with an acceptance range of ±5 ppm, and the production ion (PI), obtained from collision-induced dissociation (50 eV) (In source CID), were used for analyte identification. The values monitored for the analytes were as follows: 286.14377 (EM) (PI: 201.09101, 229.08592, 183.08044) for morphine; 300.15942 (EM) (PI: 215.10666, 243.10157, 199.07536) for codeine; 310.21654 (EM) (PI: 105.03349, 219.11683, 195.11683) for methadone; 278.19033(EM) (PI: 234.12773, 249.15120, 186.12773) for EDDP; 354.24276 (EM) (PI:167.08553, 91.05423, 105.06988) for SKF (IS).

2.4.2. Cocaine extraction and analysis

For cocaine and its metabolites analysis, each dental tissue sample was extracted and analysed according to the method previously validated by Ottaviani et al. for teeth [33]. Validation parameters used were as follow: LOQ was set at 0.05 ng/mg for both cocaine and benzoylecgonine; calibration curves, with linearity range of 0.05–2 ng/mg, had an R2 of 0.99 for each substance; precision values (%CV) were lower than 14.8 %.

Samples underwent also UHPLC analysis for search of cocaine and its metabolites.

After an overnight incubation at 50°C with HCl 0.1 M, all dental tissue samples were extracted with the SPE technique. The eluates intended first for analysis with ultra high performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC/HR-MS) were evaporated, resuspended with 50 microliter of phase B (MeOH+0.1 % FA) and 5 microliters were injected. The analytical conditions were the same used for opiates analysis. The values monitored for the analytes were as follows: 304.15433 (EM) (PI: 182.11756, 150.09134, 105.03349) for cocaine; 290.13868 (EM) (PI: 168.10191, 105.03349, 150.09134) for benzoylecgonine; 354.24276 (EM) (PI:167.08553, 91.05423, 105.06988) for SKF (IS). The remaining samples, intended for analysis with gas chromatography coupled with mass spectrometry (GC-MS), were completely evaporated and then derivatized with 30 ml of MSTFA at 60°C for 20 minutes. For the analyses, 1 microliter of the derivatized eluate was injected into the ISQ single quadrupole GC-MS (Thermo Scientific™, Waltham, MA, USA). The column used was a capillary (ZB 5 MS 30 m X 0.25 mm×0.25 µm); helium as carrier gas at a flow rate of 1.0 ml/min. The injection mode was splitless; the acquisition mode was Full Scan mode and the mass spectra range was 40–500. The values of the ions monitored were the following: *m/z* 82, 182, 303, 94 for cocaine, 82, 240, 105, 122 for benzoylecgonine and 86, 99, 165 for SKF (IS). The temperature program for cocaine detection in GC-MS was: 100°C [1 min], 220°C [40°C/min-1 min], and 320°C [20°C/min-6 min].

No systematic quantification of the substances detected in the mineralized tissues was possible.

The same method of preparation and analyses were applied to the negative-control case.

3. Results

A total sample of 10 PM teeth was collected and analysed with the new technique of dental tissue separation.

The applied methods [33,34] confirmed high sensitivity for the detection of substances even for minimal quantities of samples (in the order of 0.001 gr).

The subject who tested negative for substance intake (negative control case) confirmed negative results on classic matrices (hair and peripheral blood) and on different dental tissues (enamel, dentin, and pulp).

Substances most involved in drug-related deaths were opioids and cocaine [Tables 1–3].

4. Discussion

The background for this research is the need to provide information regarding the degree of assimilation of different drugs/substances/metabolites at the level of the enamel, root/coronal dentin and pulp, distinguishing the reliable substrate that can be applied for the diagnosis of acute overdose or chronic intoxication. Firstly, this pilot research developed a method to separately analyse enamel, peripheral and central coronal dentin, root dentin, and pulp, with the primary aim of investigating whether the substances present in blood and hair could be detected respectively in dental pulps and hard tissues (dentin and/or enamel) in cases of death due to overdose/acute intoxication and history of chronic substance abuse. The study is addressed to explore the mechanism and sites of incorporation of the substances within the dental tissues. Given the different intake methods (e.g. orally or intravenously), and the chemical-physical nature of the assumed substances, understanding the mechanism, the time, and sites of distribution and accumulation of substances into dental tissues could result in a reliable tool for forensic investigations.

According to previous literature, substances and drugs can be vehiculated and adsorbed by mineralised dental tissues, mainly dentin, through two different ways with a great interpretative difference for forensic field (Fig. 1).

The first hypothesis considers that substances are vehiculated by the peripheral systemic blood, which, through the apical foramen, vascularizes the dental pulp and cells (odontoblasts) which metabolize and deposit the secondary dentin for the entire individual life or which filter the substances within the dental tubules. This possible mechanism is supported by the studies conducted on tetracyclines, an antibiotic widely used in cases of allergies to classic antibiotics. The incorporation of these drugs within the mineral structures of the dentin in formation is a well known condition which leads to a banded pigmentation of teeth on which the classic whitening methods are ineffective, suggesting that the deposition of xenobiotics occurs mainly at level of the inner layers (dentin) [41–43]. Klima et al. [31] hypothesised that the assimilation of toxic substances in dentin occurs mainly through the blood supply by the pulp blood vessels, whilst the dental enamel is not affected as well it

Table 1
Group of subjects taking only Cocaine: comparison of the results of different dental tissues with classic matrices [blood/hair].

Cocaine			
Sample	**Qty**	**Analyses**	**Results**
ILE 1			
Hair	60 mg	GC-MS	COCAINE, BENZOYLECGONINE
Peripheral Blood	1 ml	GC-MS	COCAINE 1100 ng/ml, BENZOYLECGONINE 1088 ng/ml
P	0,023 gr	GC-MS	COCAINE 24.5 ng/mg, BENZOYLECGONINE
S	0,114 gr	GC-MS	NEG
DCP	0,366 gr	GC-MS	NEG
DCC	0,022 gr	GC-MS	COCAINE 0066 ng/mg, BENZOYLECGONINE
DR	0,041 gr	GC-MS	NEG
31 T/21			
HAIR	60 mg	UHPLC/HR-MS	COCAINE
Peripheral Blood	1 ml	UHPLC/HR-MS	COCAINE 780 ng/ml, BENZOYLECGONINE
P	0,01 gr	UHPLC/HR-MS	COCAINE 2,78 ng/mg, BENZOYLECGONINE
S	0,176 gr	UHPLC/HR-MS	NEG
DCP	0,041 gr	UHPLC/HR-MS	NEG
DCC	0,029 gr	UHPLC/HR-MS	NEG
DR	0,25 gr	UHPLC/HR-MS	COCAINE 1,26 ng/mg, BENZOYLECGONINE
26 T/22			
HAIR	60 mg	UHPLC/HR-MS	NEG
Peripheral Blood	1 ml	UHPLC/HR-MS	BENZOYLECGONINE 500 ng/ml
P	0,003 gr	UHPLC/HR-MS	BENZOYLECGONINE 1,52 ng/mg
S	0,02 gr	UHPLC/HR-MS	NEG
DCP	0,132 gr	UHPLC/HR-MS	NEG
DCC	0,015 gr	UHPLC/HR-MS	NEG
DR	0,106 gr	UHPLC/HR-MS	BENZOYLECGONINE

Table 2
Group of subjects taking only Opioids: comparison of the results of different dental tissues with classic matrices [blood/hair].

Opioids			
Sample	**Qty**	**Analyses**	**Results**
25T/22			
HAIR	60 mg	UHPLC/HR-MS	METHADONE, EDDP, MORPHINE, CODEINE
Peripheral Blood	1 ml	UHPLC/HR-MS	NEG
P	0,001 gr	UHPLC/HR-MS	METHADONE, EDDP
S	0,027 gr	UHPLC/HR-MS	METHADONE
DCP	0,158 gr	UHPLC/HR-MS	METHADONE
DCC	0,002 gr	UHPLC/HR-MS	METHADONE, EDDP
DR	0,302 gr	UHPLC/HR-MS	METHADONE
1 T/21			
HAIR	60 mg	UHPLC/HR-MS	NEG
Peripheral Blood	1 ml	UHPLC/HR-MS	METHADONE 1800 ng/ml, EDDP 472 ng/ml, MORPHINE 2538 ng/ml
P	0,027 gr	UHPLC/HR-MS	METHADONE, EDDP
S	0,026 gr	UHPLC/HR-MS	METHADONE
DCP	0,071 gr	UHPLC/HR-MS	METHADONE EDDP
DCC	0,028 gr	UHPLC/HR-MS	METHADONE
DR	0,086 gr	UHPLC/HR-MS	METHADONE
36 T/22			
Hair	60 mg		NEG
Peripheral Blood	1 ml	UHPLC/HR-MS	MORPHINE 75 ng/ml, CODEINE,
P	0,01 gr	UHPLC/HR-MS	MORPHINE 0475 ng/mg, CODEINE 0,02 ng/mg, METHADONE, EDDP
S	0,038 gr	UHPLC/HR-MS	METHADONE
DCP	0,239 gr	UHPLC/HR-MS	NEG
DCC	0,053 gr	UHPLC/HR-MS	NEG
DR	0,195 gr	UHPLC/HR-MS	METHADONE

Table 3
Group of subjects taking both Cocaine and Opioids: comparison of the results of different dental tissues with classic matrices [blood/hair].

Cocaine/Opioids			
Sample	**Qty**	**Analyses**	**Results**
ILE 4			
HAIR	60 mg	GC-MS	COCAINE, BENZOYLECGONINE
Peripheral Blood	1 ml	UHPLC/HR-MS	MORPHINE 220 ng/ml, CODEINE 46 ng/ml, 6-MAM 8 ng/ml, COCAINE 1,7 ng/ml, BENZOYLECGONINE 1000 ng/ml,
P	0,01 gr	GC-MS e UHPLC/HR-MS	NEG
S	0,059 gr	GC-MS e UHPLC/HR-MS	METHADONE
DCP	0,239 gr	GC-MS e UHPLC/HR-MS	METHADONE
DCC	0,09 gr	GC-MS e UHPLC/HR-MS	NEG
DR	0,298 gr	GC-MS e UHPLC/HR-MS	METHADONE
6 T/22			
HAIR	60 mg	UHPLC/HR-MS	COCAINE, BENZOILECGONINA, MORPHINE, 6-MAM
Peripheral Blood	1 ml	UHPLC/HR-MS	COCAINE 5 ng /ml, BENZOYLECGONINE 1590 ng/ml, MORPHINE 280 ng/ml
P	0,044 gr	UHPLC/HR-MS	EDDP
S	0,064 gr	UHPLC/HR-MS	EDDP
DCP	0,472 gr	UHPLC/HR-MS	EDDP
DCC	0,106 gr	UHPLC/HR-MS	EDDP
DR	0,398 gr	UHPLC/HR-MS	NEG
ILE 3			
HAIR	60 mg		MORPHINE, CODEINE, 6-MAM, COCAINE, BENZOYLECGONINE
Peripheral Blood	1 ml	UHPLC/HR-MS	MORPHINE 700 ng/ml, CODEINE 8 ng/ml, 6-MAM, COCAINE 4,3 ng/ml, BENZOYLECGONINE
P	0,0090 gr	UHPLC/HR-MS	NEG
S	0,0872 gr	UHPLC/HR-MS	NEG
DCP	0,4219 gr	UHPLC/HR-MS	NEG
DCC	0,0241 gr	UHPLC/HR-MS	6-MAM
DR	0,2207 gr	UHPLC/HR-MS	NEG

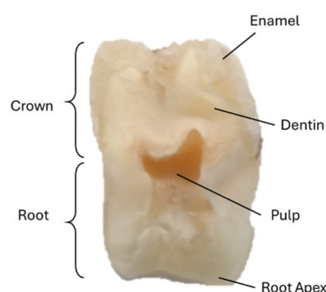

Fig. 1. Cross-section of a molar tooth with different dental tissues at the crown and root level.

represents a protective barrier from contamination by molecules coming from external environment and vehiculated by the saliva.

On the contrary, the second hypothesis considers the possible contamination from the oral cavity through the enamel which can increase its porosity as a result of severe wear, salivary pH decrease, or the exposure of the cementoenamel junction. In this sense, Klima et al. (2016) [31] reported a higher concentration of substances detectable in teeth, in case of decayed teeth in which enamel prisms and dentinal hydroxyapatite destroyed by carious are supposed to allow the passage of bacteria and bacterial toxins from the oral cavity in to the dental pulp.

Preliminary results of the present research are obtained from real autopsy cases, therefore without the control and selection of the substances of abuse and the methods of intake. The main detected substances were cocaine, opioids, and their metabolites (Tables 1–3). The first finding is the repeatability of the separation of dental matrices (pulps, dentin, and enamel) with the innovative technique, and the reliability of the analysis methods by Ottaviani et al. [33] and Buratti et al. [34] on pulps and mineralized tissues in the determination of opiates, cocaine, and their respective metabolites even on very small quantities of tissue (up to a minimum of 0.001 gr). Analyses can be easily performed in most toxicological laboratories with accessible equipment and are suitable for different drugs in dental tissues and thus reliable for forensic post-mortem cases.

The control sample taken from a subject who was negative for substance intake, confirmed the basic absence of substances in dental tissue, supporting the hypothesis of a correlation between systemic intake and accumulation in dental matrices.

For two cases, ILE3 and ILE4 (Table 3), no morphine, codeine, cocaine and benzoylecgonine, were detected in pulp samples differently from the respective blood samples, in which these substances were found. As shown in Table 1 (cocaine), there is a qualitative but not quantitative correspondence between the substances detectable in the peripheral blood and in the pulp and the pulpal concentrations are lower than blood. For instance, the sample ILE1 (Table 1) has concentrations of cocaine of 1100 ng/ml and 1088 ng/ml of benzoylecgonine in blood compared to cocaine 24.5 ng/mg and traces of benzoylecgonine in dental pulp. This result could be plausibly due to the quantity intake influencing the low blood concentration, and much lower in pulp microcirculation, thereby in pulps substance concentration could decrease below the minimum quantity of detectability. This hypothesis seems to be supported by the blood results of the ILE3 sample, with a quantity of cocaine actually low (4.3 ng/ml) as well as for benzoylecgonine (traces), and for the ILE4 sample (cocaine 1.7 ng/ml) (Table 3). However, a minimum quantity of detectability (threshold) for pulps is not measurable in this study since is based on 9 samples of drug-related deaths. Further research is needed to increase the sample size and then correlate quantitative results between pulps and blood.

In the blood of 31 T/21 and 26 T/22 samples in Table 1 were detected traces and 500 ng/ml of benzoylecgonine respectively with a qualitative correspondence of these substances in pulps; on the contrary, the blood of ILE4 sample showed 1000 ng/ml of benzoylecgonine (Table 3) with no qualitative correspondence in pulp. This different "behaviour" could be explained by the different assumption groups: 31 T/21 and 26 T/22 samples belonged to subjects who took only cocaine in life; whilst, ILE4 took cocaine in association with opioids. Table 1 shows cases in which the substance intake was only cocaine and for all samples it was found a qualitative correlation between substance (cocaine) and its metabolites (benzoylecgonine) in the dental pulp and in peripheral blood. On the contrary, the lowest concentration rates in pulps decrease or increase in a manner apparently directly proportional to the decrease or increase of the concentration rates of the substance and its metabolites detected in the blood. This suggests the possibility of a measurable correlation between the different matrices and therefore applicable for the reconstruction of the subject's conditions in the acute phase of intoxication. However, it is necessary to collect a larger number of homogeneous samples taking only cocaine to build a reliable

regression between concentration rates of the substance separately measured in blood and in pulp. This result seems to support the conclusion that in the pulp can be retrieved the same substances circulating in blood and the lower concentrations in pulps seem in line with the anatomy of the dental microcirculation and with the quantity of sample available for the analysis (approximately 0.02–0.003 g). ILE1 and 31 T/21 samples (Table 1) which came from cases of cocaine chronic assumption, showed an interesting qualitative correlation between substances detected both in hairs and in secondary dentin, whilst the dental enamel resulted completely negative.

Looking at the different assumption ways for cocaine, both local and bloodstream absorption must be considered. Usually, oral administration of cocaine consists of a short and occasional local application onto the gingiva to test its quality, whilst the most diffused method of assumption is crack-cocaine smoking with a chronic addiction due to the "craving" effect [44,45]. Nonetheless, bruxism and the reduction of saliva pH after oral or nasal application are common complications leading to dental attrition and erosion [44], which easily compromise the integrity of the enamel of addicted subjects. Since in none of the 3 cases in Table 1 the presence of cocaine in the enamel was detected, the possible oral intake does not emerge as sufficient cause an external contamination of the tooth, suggesting that the topographic distribution of cocaine from the most apical (radicular) to the most coronal areas of secondary dentin can be attributed to the active metabolism of the odontoblastic cells from the bloodstream concentrations due to chronic addiction confirmed by the results of hair.

Moreover, in 26 T/22 sample (Table 1), the substances were found in the dental hard tissues but not in the hair. This finding allows to verify the hypothesis that the drug intake window is much wider for dentin than for the hair. In fact the secondary dentin is deposited throughout life starting from the moment of complete formation of the teeth (from 12 to 23 years depending from the tooth and sex of the subject), therefore the chronic accumulation of substances enables to detect substances accumulated years before in a deep layer of dentin or at least a very long-term analysis for the drug addiction. These findings are in line with those obtained by Klima et al. in a recent article of 2023 [46], where they studied the deposition of some substances of abuse (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), morphine, codeine, 6-acetylmorphine (6-MAM), cocaine and benzoylecgonine (BE)) in dentin by simulating various *in vitro* models to investigate the relationship between the amount of xenobiotic deposited and the contact time, as well as the properties of the studied substances. They concluded that the deposition of the substances probably depends on its physicochemical properties, the duration of contact and the concentrations present in the blood and oral cavity. They noted that higher concentrations of the substance in teeth may result from frequent or longer use of the drug, but the study did not consider the enamel and its specific features.

As a novel evidence, the present study demonstrated that the dental enamel is not immune to all the molecules present in the oral cavity. Table 2 reports the results of subjects intaking opioids, and in particular methadone. Compared to cocaine, methadone and its metabolites have been found in pulp, and dentin, but also in dental enamel. Moreover methadone and its metabolites were found in pulp, dentin and enamel even in cases for which they were not detectable in peripheral blood or hair. These findings strongly suggest a contamination from the oral cavity of dental tissues and especially enamel, facilitated by the oral assumption of methadone, that is administered as syrups that contain sugars or sucrose. Usually, heroin users, although injectors, show poor oral health with progressive caries on smooth and cervical surfaces and periodontal diseases due to a salivary hypofunction leading to xerostomia and mucosal infections, up to necrotizing gingivitis [44]. Methadone is a synthetic opioid widely used to manage heroin addiction which, on the contrary, is typically taken orally: the high sugar acidic content along with the suppression effect on salivary secretion lead to tooth decay, erosion and xerostomia. These conditions are aggravated when patients on therapy keep sugary syrups in their mouth (under the tongue) to increase its absorption time or regurgitate it for later injection or illegal sale [44], whilst sugar-free solutions seem to reduce the risk of dental caries [47]. The method of intake and the duration of oral contact with the substances, emerges as a crucial factor that can affect the penetration of substances into enamel and other underlying dental tissues, especially when the substance is combined with sugars that can induce a demineralisation of the enamel layer, due to bacterial production of acid (cariogenic mechanism). This interpretative approach fits perfectly also for subjects who took methadone combined with cocaine (Table 3), in which the distribution of cocaine is apparently chaotic and random compared to Table 1. In fact, ILE4 sample (Table 3) highlights the presence of methadone in most hard dental tissues (enamel, peripheral dentin and root dentin), which is not present either in the blood or in the hair. This tends in general to exclude that deposition of the methadone occurred via bloodstream. Moreover, sample 6 T/22 highlights the presence of EDDP (metabolite of methadone) in some dental tissues from the innermost to the outermost (pulp, central coronal dentin, peripheral coronal dentin and enamel) but both EDDP and methadone are absent in hair and blood. Considering that EDDP is produced by active metabolism, this suggests that it was assumed some months before the death since neither methadone nor EDDP is no longer detectable in blood and hair. Hence, it can be concluded that methadone was metabolised in life and the produced EDDP accumulated in all dental inner tissues up to the enamel, in which these substances penetrated for the higher permeability due to a compromised external layer.

In conclusion, the presence of methadone into the enamel of our dental samples can only be attributed to the route of intake (orally), to the typical association with sugar which determines the increase in the permeability of the external enamel, and to the prolonged oral exposure of the substance to increase its effect [33,48–50]. Therefore, it is possible to consider that in the case of intact enamel, intravenous intake, or short duration of the oral contact with substances (e.g. cocaine) there is no possibility of an external contamination and the concentrations detectable in dental tissues correspond to the systemic concentrations of the subject; on the contrary, in case of damaged enamel, xerostomia, lower pH salivary, tooth wear, or sugar the concentrations detected in the tooth are probably due to a direct contamination from oral external fluids.

In case of heroine assumption (Table2–3), it emerged that 25 T/22 hair sample, 1 T/21 blood sample, and 36 T/22 blood sample (Table 2) revealed the presence of morphine and 6-MAM, as well as ILE4 blood sample, 6 T/22 blood and hair samples, and ILE3 hair and blood (Table 3). Therefore, apart from pulp sample of 36 T/22 (Table 2), morphine was never detected in dental tissues and only in ILE3 sample was found 6-MAM in the central dentin. These results seems to be not in line with those obtained by previous studies [30–33] in which morphine was detected in the most analysed teeth, even different tissues were not analysed separately as performed in the present research. Klima et al. [31] detected a correspondence between time and quantity of substance in hair blood and mineralized dental tissues and pulp, finding the absence of morphine in dental hard tissues in case of presence in hair indicating occasional consumption of heroine and the absence of morphine in pulps in case of a recent assumption of heroin in which the concentration of morphine in femoral blood resulted lethal. Following an intravenous administration of 120–450 mg heroin, 6-MAM is mostly metabolized in 0.3–2.7 min after injection followed by morphine that is metabolized within 3.6–7.8 min [51].

Hence, the presence/absence of heroin and its metabolites in the different dental areas seems to be influenced by the constant abuse of heroin and the survival time of the subject before death.

The main limitations of the study consist in the low availability of dental samples and substances of abuse. Results are still limited to be generalized and further researches are necessary to repeat the analyses on multiple samples from the same subject for each detectable substance

and on different subjects taking the same substance. Another significant limitation is that the experimental study was conducted on real autopsies, with limited history available about the quantity and the different methods of assumption for different substances of abuse.

5. Conclusions

Dental matrix is different from any other matrix known for post-mortem toxicology since preliminary results suggest the usefulness of teeth in post-mortem investigations both for acute intoxication (dental pulp) and for chronic intake (mineralized tissues - enamel and/or dentin), as a unique matrix for both acute and chronic assumptions of drugs. Except for methadone, no other drugs were detected in the enamel.

The drug intake window is much wider for teeth than for the hair and the chronic accumulation of substances in deep dental dentin enables to detect a very long-term (e.g. years) analysis for the drug addiction.

The main finding is that the method of intake and the duration of oral contact with the substances, emerges as a crucial factor that can affect the penetration of substances into enamel and other underlying dental tissues, especially for methadone that is administered as syrups with sugars that can induce a demineralisation of the enamel layer due to acid produced by oral bacteria as it happens in dental caries.

In the absence of association with methadone, cocaine appears to accumulate in the secondary dentin via the bloodstream of the dental pulp vessels and shows qualitative correspondence between the determination of substance in the pulp and in the peripheral blood. This result supports the conclusion that the substances circulating in blood can be retrieved in the dental pulp, but a quantitative correlation between the two matrices for acute intoxication (dental pulps and peripheral blood) requires a larger sample to yield a valid regression formula.

The innovative method for the separation of dental tissues is promising for the study of the different mechanism, pathways, speeds and sites of incorporation of xenobiotics in different dental tissues.

Further investigations are needed for different drugs and in samples with known and detailed history of abuse to better understand the relevance and reliability of dental matrices as a possible new forensic tool.

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Declaration of interest

None.

CRediT authorship contribution statement

Martina Focardi: Resources. **Alice Cerioni:** Formal analysis. **Gianmario Mietti:** Formal analysis. **Marta Cippitelli:** Writing – review & editing, Supervision, Resources, Investigation, Formal analysis. **Erika Buratti:** Formal analysis. **Vilma Pinchi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Ilenia Bianchi:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Rino Froldi:** Methodology, Investigation. **Mariano Cingolani:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Rossella Grifoni:** Resources. **Roberto Scendoni:** Resources.

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.

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