Balkan Academy of Forensic Sciences

Understanding Crime Through Forensic Sciences

Edited by:
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FOREWORD

Forensic science is a field that rapidly and continuously evolving. The technology today promises even the full visual characteristics of a suspect in a crime. Good news is that this advancement may also potentially be used in the development of strategies for crime prevention. The accelerating power of computer sciences has also played and will continue to play a very important role in the field. Apart from huge data storage capacity, high resolution images and computational error rates, validation methods and various related algorithms are already present and we expect further progress.

Within this framework, academic cooperation and partnership is essential. One of the best methods to achieve this is by the organization of scientific meetings where it is possible to share useful new information, ideas and develop science communication. Scientific meetings, apart from forming a forum for scientists to present their research, it can be an opportunity for brainstorming, networking and developing connections that can lead to innovativeness.

Regional scientific cooperation in Balkans on the other hand is extremely important because it can lead to scholarly collaboration and knowledge sharing thereby bridging the knowledge gaps the are present in the area.

We believe that this initiative may lead to the coordination of major projects that will be invaluable for the scientific community.

Ersi Kalfoglou
President of BAFS

Nermin Sarajlic
Co-President of BAFS

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PREFACE

A group of distinguished scientists from the Balkan region and not only, realized the importance of bringing Balkan scientists together and they decided to organize the foundation of the Academy 20 years ago. It was thanks to them that the academy expressed the unity of feelings, attitudes, and hopes in the future for real scientific cooperation from the beginning.

We appreciate their roles and salute the memory of those that are not with us anymore.

With the intention of keeping the effort of the founders alive, we are continuing to organize the meetings and bring together the scientific community for the benefit of Forensic Sciences.

The aim of the BAFS is to deal with all scientific, educational and professional matters pertaining to this discipline on an international level. The objectives of the Academy are to promote education for and research in the forensic sciences, to encourage the study, to improve the practice, to elevate the standards and to advance the cause of the Forensic Sciences. It is also to promote interdisciplinary communications and to plan, organize and administer meetings and other projects for the stimulation of these and related purposes.

We have promised to publish selected papers of the last meeting in a book and this is how this publication has been realized. You will find interesting topics coming from diverse fields of forensic sciences and I hope that you will enjoy it.

We expect that this effort will stimulate scientific debate, increase the network between scientists and stimulate further research.

Sotirios Kalfoglou

Dilek Salkım İşlek

FORENSIC APPLICATIONS OF LASER-INDUCED BREAKDOWN SPECTROSCOPY (LIBS)

Eda Kiriş, Dilek Salkım İşlek, Emel Hülya Yükseloğlu

Forensic Science can be generally defined as the application of science to law in solving cases. Forensic scientists use multidisciplinary (physics, chemistry, biology, mathematics, medicine, psychology, sociology, etc.) scientific techniques to analyze physical evidence in order to found or externalize a relationship between a suspect and the crime scene. It is now possible to identify a sample and determine whether two or more objects share a common origin using a wide range of analytical techniques. Laser-Induced Breakdown Spectroscopy (LIBS) is one of these methods. LIBS is an optical emission technique that creates a spark (i.e., breakdown) directly in/on the sample using a high peak power pulsed laser beam. This spark generates a plasma hot enough to break molecules into their component atoms and excite electrons in the plasma from the ground state to excited electronic states. As the plasma cools, excited electrons and ions return to their ground states and emit light at particular atomic wavelengths.

An ordinary LIBS set-up includes pulsed laser, focusing system, target, collection system, detection system, computer and software system (Figure-1)[1].

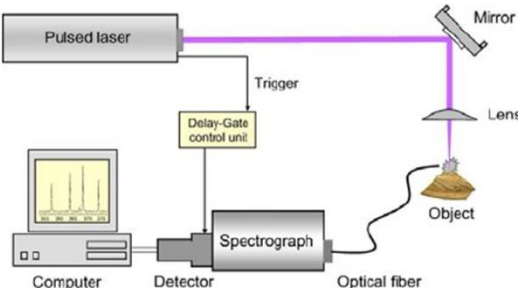

Figure 1: LIBS set-up

LIBS compares favorably to other elemental analysis techniques and provides numerous significant benefits for forensic trace evidence analysis. First of all, with a single laser pulse, all elements in the periodic table can be analyzed successively, including atoms with low atomic masses that are not detectable by traditional analytical methods. Also compared to other techniques LIBS is less expensive [2]. Because the LIBS process consumes such a small quantity of material, it is considered fundamentally non-destructive or minimally damaging. Also, No or only minor sample preparation is needed which means without the requirement to solubilize the sample, LIBS can be utilized to examine solids directly. Also, LIBS instruments can be adjusted and reinforced such that portable systems can be used to analyze evidence near or at a crime scene [3]. Lastly, The LIBS method is relatively fast; a trained operator might handle 10–20 samples in under an hour.

LIBS offers several important advantages, but it also has limitations that must be addressed when considering it as a means for forensic analysis, as with any analytical techniques. Initially, the shot-to-shot variability of LIBS data sometimes results in lower precision (about 5–20 % RSD) than other known forensic analysis methods. Secondly, LIBS has relatively high detection limits (high ppb- low ppm). As a last one spectra obtained on multiple LIBS instruments from the same sample cannot be expected to be identical [4].

Despite the fact that it is challenging to itemize all type of physical evidence, there are some materials like glass, paint, ink and paper, counterfeit currency and gunshot residue can be analyzed by LIBS.

Glass Analysis

Glass can be a valuable source of evidence, allowing police to link a suspect (or an automobile) to a burglary or hit-and-run accident. In the crime scene, glass pieces can be coming from building windows, beverage containers, automobile windows, headlights or mirrors are examined in the forensic laboratories. General glass examination is done by using atomic emission spectroscopy (AES), atomic absorption spectroscopy (AA) or inductively coupled plasma mass spectrometry (ICP-MS). However, several studies have been published on the use of LIBS to analyze glass. One of them is first paper to focus on forensic glass analysis. In this paper an automobile headlamp glass, brown beverage container glass and automobile side window float glass, was analyzed by LIBS. LIBS spectra in the Ar

atmosphere were gathered using a broadband spectrometer and Q-switched Nd:YAG laser operating at 1064 nm. Emission lines for Al, Ba, Ca, Cr, Fe, Mg, Na, Sn, Si, and Ti, which were spectrally isolated and had no obvious shoulders or overlapping peaks, were utilized. Theree spectra is different than other (Figure-2). In this way pieces of glass that was collected from crime scene can be identify which type of glass is it[5].

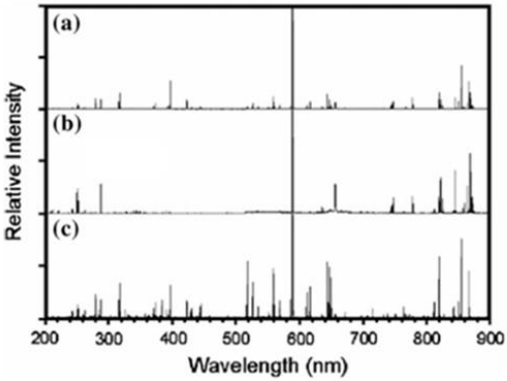

Figure 2: LIBS spectra taken from three different types of glass; a-) automobile headlamp glass, b-) Brown beverage container and c-) automobile side window float glass.

Paint Analysis

Next topic is paint. Different type of paint can use in automobile, art works or wall of houses. Paint is made up of a binder, pigments, and a variety of additives that change by type of paint. Pyrolysis gas chromatography mass spectrometry (Py-GC-MS) and Fourier transform infrared spectroscopy (FTIR) can be used to determine the organic components of a paint sample. Inorganic pigments can be identified by Raman spectroscopy and LA-ICP-MS. However, in 2000 Stratoudaki published a paper about artwork forgery. In this paper several icons were examined. First work is on a late 16th century (egg tempera on wood) icon, portraying Virgin Mary holding Jesus. They examined white, green and red paint areas (Figure-3).

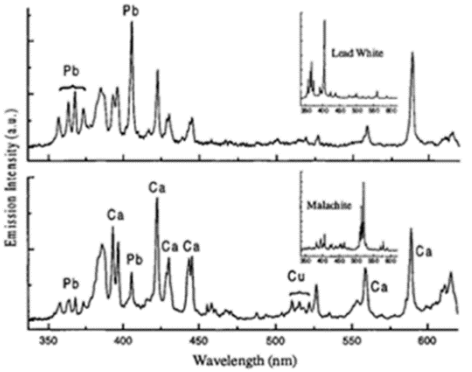

Figure 3: LIBS spectra white (top) and green(bottom) paint of portraying Virgin Mary holding Jesus.

Secondly a mid-19th century icon (egg tempera on silver foil) of Saint Nicholas was also examined. After first shot zinc spectrum was obtained. However, after three shots lead spectrum was appeared. It was discovered that the original white pigment used on this icon was lead white. Finally, only calcium was found after forty pulses on the same location, showing that the preparation layer (most likely calcite or gypsum) had been obtained (Figure-4).

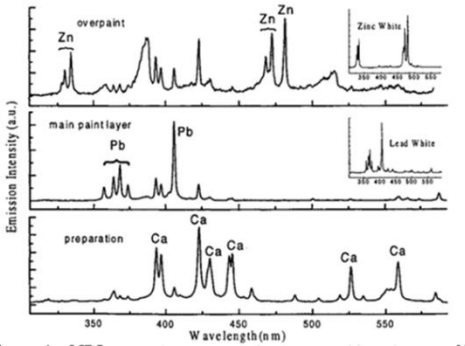

Figure-4: LIBS spectra from selected point on a White paint area of Saint Nicholas icon revealing the thin overpaint (2nd pulse), the original paint (5th pulse) and the preparation (40th pulse).

A 16th century icon, The Annunciation, was also examined. The mercury peaks found in the red paint confirmed that it was cinnabar (HgS). When a yellow pigment was investigated, the LIBS spectrum

became slightly more complicated. Atomic emission lines due to lead, chromium and calcium were observed. These findings suggest that lead chromate (chrome yellow), has been used on this icon (Figure-5). Given that chrome yellow was introduced in the mid-eighteenth century and the symbol dates from the sixteenth century, the originality of this yellow paint has been questioned [6].

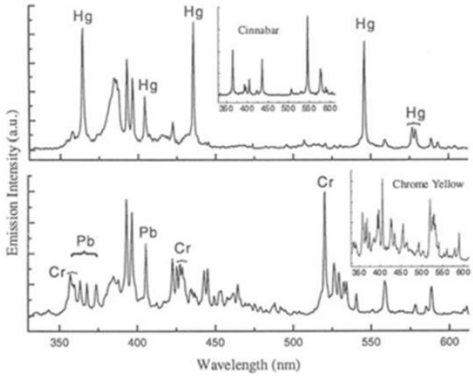

Figure 5: LIBS spectra of red (top) and yellow (bottom)paint layer of The Annunciation icon.

Ink Analysis

Last subject is ink. Ink and paper generally is a evidence for crime that falsified business transactions, counterfeit currency. Because of that comparison and/or identification of inks based on their chemical composition is one of the most essential parts of questioned document analysis. Writing inks are composed of colorants (dye and/or pigment), a solvent known as a "vehicle" and additives (responsible for the physicochemical properties of inks). Ink examination can be done by using TLC or laser desorption ionization mass spectrometry (LDI-MS). However, with developing technology ink can be analyzed easily by LIBS. In paper 2013, 34 blue, 30 black and 21 red writing instruments of various types (40 ballpoint pens, 29 gel pens, 6 porous point pens and 10 rollerball pens) and different brands was analyzed. The spectrum of theree of them are different from each other (Figure-6). On the other hand, inter laboratory test was done. Two different person was chosen and then want them write suspect1 and suspect 2 with unknown pens. Differ than two-person one researcher write reference. Looking for answers to two questions, first one Is 'Suspect 1' writing ink the same

as 'Reference' writing ink? and second one Is 'Suspect 2' writing ink the same as 'Reference' writing ink? After LIBS analysis, theree spectrum was examined. According to spectrum theree of writing inks are different from each other (Figure-6). The results were verified as correct [7].

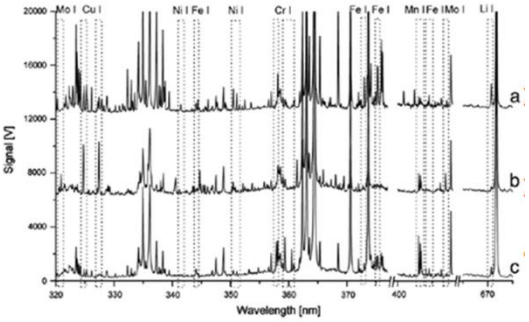

Figure 6: LIBS spectra of inks from a-) a blue ballpoint pen, b-)a black ballpoint pen, c-)a red gel pen

As a result, LIBS can be analyzed any kind of evidence such as ink, paint, glass and etc. Apart from the mentioned articles, LIBS contributes to the cultural heritage by analyzing bones and teeth. Also, LIBS can be used for soil analysis. After death searching for heavy metal on soil can be possible by LIBS. On the other hand a customized instrument has been designed for LIBS for fast characterization of the composition of excipients and pharmaceutical substance in tablets. Since LIBS has many benefits, it should be used in every criminal laboratory.

Without the need for sample preparation, LIBS proposes the appealing option of fast operating, least damaging elemental analysis directly on virtually any sort of material relevant to criminal proceeding. The technology is generally capable of purchasing and maintain, and the method is relatively simple to learn. LIBS have better ability to discriminate comparable to more expensive other instruments such as LA-ICP-MS in many applications, according to careful tests utilizing optimum parameters. The technique's real and perceived effectiveness will be strengthened by the advancement of analysis standards, the application of periodic blind testing and the routine round-robin type testing in operational forensic laboratories. This is necessary to make sure that the requirements set by Frye and Daubert are followed when applying LIBS to forensic evidence.

In conclusion, LIBS enables concurrently analysis of all elements in the periodic table. Also, LIBS less expensive than other analytical techniques. On the other hand, it is non-destructive or minimally damaging which is important for if there are lack of evidence. It saves time because of no or only minor sample preparation is needed. The most important thing is it is a portable system which can be into the field for analysis of evidence at or near a crime scene. Because of these advantages LIBS should be in all forensic laboratories.

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LABORATORY ANALYSIS OF USED ORAL FLUID DRUG TESTING ON-SITE DRÄGER DEVICES

Silviya Stoykova, Vasil Atanasov

Oral fluid (OF) is a sample of increasing interest as an alternative matrix to the blood, especially in screening on-site testing of drivers of motor vehicles, suspected in illicit drugs intake. The main advantages of OF over the common biological matrices for toxicological analysis – blood and urine, include: (i) readily available sample - easy, painless, non-invasive and undemanding specialized personnel collection which can be performed at any place; (ii) supervised sampling without person's embarrassment, i.e. more difficult to adulterate and to substitute sample; (iii) pharmacologically active (parent) drug identification - drugs in OF appear due to passive membrane diffusion, active secretion or ultrafiltration which processes require compounds to be lipid-soluble, non-ionized and unbound to proteins (free form); (iv) the concentration of a detectable substance in OF is generally commensurable to the free fraction of the drug present in blood - the detection window for drugs of abuse (DoA) in OF approximates that in blood but also might be influenced by factors as administration route, pH medium, solubility, flow rate, frequency of abuse, etc.; (v) recent drug usage – the statement that concentration of drugs in OF should be equivalent to the free pharmacologically active substance in the blood makes it possible to correlate the concentration of the substance and its pharmacological effects on the individual although that is oversimplification. However, OF also has some disadvantages such as limited sample volume, uncertain composition, etc. which in practice represent the challenges in toxicological analysis. In forensic toxicology such kind of sample is greatly appreciated because allowed rapid qualitative analysis permitting the performance of so-called "controlled substances tests" of drivers. The roadside OF testing is currently based on immunoassay using various technical devices. Nevertheless, in case of positive OF screening result, still blood remains a preferable matrix for confirmatory drug analysis. Confirmation of preliminary on-site testing results is performed by hyphenated analytical

technique that combines the separation properties of gas or liquid chromatography with the detection feature of mass spectrometry to identify different substances.

In Bulgaria, a "*per se*" legislation (zero-tolerance law) for drugged drivers exists i.e., it is illegal to drive with any detectable amount of illicit DoA in the blood. This facilitates the implementation of legislation, since the prosecution does not have to prove that driver was impaired. The use of DoA has to be evidenced by results from technical on-site OF devices and/or from laboratory blood confirmatory analysis. The current practice in the country (since 2011) for drivers, suspected in illicit drugs intake, consists in OF immunoassay testing using on-site devices as Dräger DrugTest 5000 or Dräger DrugCheck 3000 (Fig. 1).

Figure 1: Technical on-site devices for OF screening of drugged drivers a. Dräger DrugTest 5000 and b. Dräger DrugCheck 3000.

In case of positive result, confirmation has to be done in blood. Driver must go through medical examination during which the physician describes observable symptoms of illicit drugs usage (behavior, general mental and somatic condition, any signs of abstinence, etc.) as well as if any other medicines were prescribed to the person. Then blood and urine samples are collected and sent for toxicological analysis. When driver denial to give a blood sample, then result based on preliminary testing should be accepted. However, in some cases the judiciary demand to evidence these results performing confirmation of drug presence in residual OF on the swab of Dräger device.

In the current study, 113 on-site drug testing devices were presented to confirm a preliminary positive result for illicit drug usage. The aim of this study is to evaluate the prevalence of confirmed samples for conventional DoA – tetrahydrocannabinol (THC), amphetamine (AMP)/ methamphetamine (MET), cocaine (COC) and opiates (OPI), taking into account the elapsed time from the time of the preliminary inspection to the laboratory analysis, as well as the storage conditions of the technical device.

Chemicals and Reagents

All reagents were of analytical grade and all solvents used - of chromatographic grade. Hydrochloric acid (HCl), sodium hydroxide (NaOH), anhydrous sodium sulfate (Na_2SO_4), potassium hydroxide (KOH), sodium hydrogen carbonate ($NaHCO_3$), acetic anhydride (Ac_2O), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), sodium acetate (AcONa), acetic acid (AcOH), *iso*-propanol (*i*-PrOH), ammonium hydroxide (NH_4OH) and N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) were supplied from Merck (Germany). Methanol (MeOH), ethylacetate (EtOAc), anhydrous magnesium sulphate ($MgSO_4$), *tert*-butyl methyl ether (TBME) and dichloromethane (CH_2Cl_2) were purchased from PanReac AppliChem (Germany).

In all experiments deionized water (dH_2O; 18.2 MΩ•cm) was used.

Materials

113 on-site devices for drug roadside testing in OF were submitted by relevant authorities for confirmatory analysis. In all cases driver refusal to give a blood specimen for forensic toxicology examination. Preliminary results are reported as positive relatively to a cut-offs value shown in Table 1.

DoA	Dräger DrugTest 5000 Cut-offs, ng/mL	Dräger DrugCheck 3000 Cut-offs, ng/mL
THC	5, 10 or 25	15 or 40
AMP / MET	50 / 35	50 / 50
COC	20	20
OPI	20	20
BZD	15	-
MTD	20	-

Table 1: Cut-off values for different DoA in neat OF, stated in Dräger DrugTest 5000 and Dräger DrugCheck 3000 manufacturer's instructions.

The cartridges obtained were opened and the OF collector swab (Fig. 1) was transferred into test tube and was treated with 6 mL mix of dH_2O, MeOH and 1M HCl in equal amounts in order to elute efficiently drugs adsorbed onto collector pad and to convert them into corresponding water-soluble hydrochlorides (24). After sonication (20 min), the extract acquired was used as a sample in subsequent experiments.

Drugs of Abuse (DoA) Laboratory Screening Analysis – Sample Preparation

Thus, the sample, obtained after preparative processing of the specimen, undergoes suitable liquid-liquid extraction (LLE) or solid-phase extraction (SPE), depending on the preliminary results available.

THC, COC, BZD and MTD were detected using following sample-preparation protocol (24): To the 6 mL pre-treated extract 2 mL 1M NaOH was added and an extraction with 7 mL EtOAc was carried out. After vortex-mixing and centrifugation (3 000 rpm / 3 min), the organic layer was evaporated to dryness under nitrogen (N_2) and reconstituted in 50 μL EtOAc. 1 μL of the sample was used

for gas chromatography-mass spectrometry (GC-MS) analysis.

AMP and MET were assayed using LLE as follows (25): 500 μL buffer (10 M KOH:saturated $NaHCO_3$, 3:17 v:v) were mixed with pre-treated extract (6 mL). Then, 5 mL TBME and 150 μL Ac_2O were added and the sample were vortexed immediately for 2 min. After centrifugation (3 000 rpm / 5 min), the upper organic layer was transferred to a clean tube and concentrated under N_2 to a final volume 50 μL. 1 μL was injected into the GC-MS.

The identification of OPI was performed after SPE on Strata Screen C (Phenomenex, USA) according to manufacturer's instructions. In brief, the procedure consists of: 1) condition with 2 mL MeOH and 2 mL 100 mM phosphate buffer pH 6.0, 2) loading 6 mL pre-treated extract pre-mixed with 4 mL 100 mM phosphate buffer pH 6.0, 3) washing sequentially with 3 mL dH_2O, 3 mL 100 mM acetic buffer pH 4.5, 2 mL MeOH and after dryness the column under full vacuum 4) eluting with 3 mL of CH_2Cl_2:*i*-PrOH:NH_4OH (78:20:2 v/v/v) into clear test tube. The mixed solvent was evaporated under a gentle stream of N_2 and the residue was derivatized with 30 μL BSTFA and 30 μL EtOAc for 20 minutes at 70 °C. The injection volume was 1 μL of the sample.

The limits of detection (LOD) of GC-MS screened DoA were shown in Table 2.

DoA	GC-MS LOD, ng/mL
THC	10
AMP / MET	10
COC	20
OPI	50
BZD	10
MTD	20

Table 2: Limits of detection (LOD) of DoA, analyzed during GC-MS screening.

Instrumental analysis – GC-MS conditions

GC-MS analyses were carried out on an Agilent 7890B GC system with 5977A MS detector equipped with autosampler G4513A and fitted with a DB-1701 capillary column (length 30 m × internal diameter 0.25 mm, 0.25 μm film thickness) (Agilent Technologies, USA). The injector was operated in splitless mode at a temperature of 270°C. Helium was used as carrier flowing at 1.5 mL/min. The oven temperature program was: 50°C for 1 min, increased to 150°C at 10°C/min, held for 1 min and then, to 280°C at 8°C/min, with a final hold time of 15 min. The transfer line temperature was 250°C. The MS was operated in positive electron ionization (EI^+), full scan mode with range of 40-550 m/z, 150°C quadrupole temperature, 230°C ion source temperature, MS selective detector interface at 300 °C and 70 eV electron energy. The data processing and instrument control were performed by Agilent MassHunter Workstation software (Agilent Technologies, USA).

On-site devices (80 of Dräger DrugTest 5000 and 33 of Dräger DrugCheck 3000) were presented in Forensic Toxicology Laboratory, Military Medical Academy – Sofia by traffic police authorities. In all of these cases, the relevant persons (6 females, 105 males and 2 with no available information about gender) had refused the subsequent confirmation of the preliminary OF immunoassay result in a blood sample by appropriate toxicological analysis. Thereon, the confirmation was performed by GC-MS screening of residual OF on collector swab of corresponding on-site device. The data, obtained from in-lab analysis, is summarized in Table 3.

DoA	On-site Dräger devices	Number of sample devices	Confirmation with the preliminary roadside result	Differences	Negative laboratory results	% Confirmation
THC	Drug Test 5000	12	6	1 (MTD)	5	50.00
	Drug Check 3000	7	1 (+AMP)	0	6	14.29
AMP / MET	Drug Test 5000	23	11	0	12	47.83
	Drug Check 3000	4	1	0	3	25.00
COC	Drug Test 5000	1	1	0	0	100.00
	Drug Check 3000	2	0	0	2	0.00
OPI	Drug Test 5000	9	3	1 (MTD)	5	33.33
	Drug Check 3000	7	3	1 (MTD)	3	42.86
BZD	Drug Test 5000	0	0	0	0	-
MTD	Drug Test 5000	1	0	0	1	-
Combined use (two or more drugs)	Drug Test 5000	32	10	12	10	31.25
	Drug Check 3000	8	1	3	4	12.50

Table 3: Percent confirmation between results obtained from roadside testing and those from laboratory GC-MS analysis of used on-site Dräger devices.

The overall prevalence of confirmed positive samples for all tested DoA was 38.75% (21.21% for the Dräger DrugTest 5000 and 23.3% for the Dräger DrugCheck 3000, respectively). The best correlation between on-site OF testing and in-lab analysis was found to be in case of THC-positive and AMP/MET-positive Dräger DrugTest 5000 – 50% and around in both. The same DoA show the worst correlation when preliminary roadside test is performed with Dräger DrugCheck 3000 – 25% or less.

In case of combined drugs usage (40 cases), the average percentage confirmation was 27.50%. Preliminary positive results for both AMP and MET (15 cases) are expected and are probably due to the fact that amphetamine is a metabolic product of methamphetamine (26). Another possible explanation could be the cross-reactivity. The next most common combination of DoA was AMP, MET and THC (11 cases). This was also not surprising, due to the ever-increasing interest in combining cannabis consumption with simultaneous use of other psychoactive compounds such as amphetamine-type stimulants (27). This fact was in agreement with previously published results (28). Other combinations were AMP,

MET and COC (4 cases); THC and OPI (2 case); AMP and OPI (1 case); AMP and BZD (1 case); THC and COC (1 case); AMP, MET and BZD (1 case); AMP, THC and OPI (1 case); AMP, THC and BZD (1 case); AMP, MET, THC and COC (1 case); AMP, MET, THC and BZD (1 case).

There are 7 cases among all on-site tested devices where no preliminary data was obtained i.e., tentative positive result from roadside testing was not specified. In only one of these cases the laboratory examination of used on-site Dräger device confirm the presence of DoA – AMP, COC and MTD were detected.

It is important to notice that pre-lab storage temperature of all tested devices is unclear. Usually, these test-cassettes are attached to relevant documentation on the case so one could assume that they are kept at room temperature.

The elapsed time between preliminary roadside test and laboratory analysis is also of paramount importance (Table 4). In current study this storage period varies widely from 5 to 573 days. In 27 cases there is no preliminary data available for the date of on-site OF test i.e., it is impossible to calculate the elapsed time between the immunoassay test and instrumental analysis.

Elapsed time	Total number of cases	Positive lab results	% Confirmation
Up to 30 days	25	12	48.00
Between 30 and 60 days	20	10	50.00
More than 60 days	41	10	24.39

Table 4: The relationship between elapsed time between roadside test and laboratory analysis and percent confirmation in these cases.

In roadside DoA testing, when immunoassay is used as a screening test for the presence of illicit drugs in OF, the positive result has to be confirmed by instrumental analytical technique (5, 22, 23). The detection and monitoring of DoA and/or their metabolites have traditionally been performed using blood (whole blood, serum, plasma). However, in cases discussed in the current study, the driver refuses blood sampling. Thus, prosecution demand to subsequent confirmatory testing of OF on already used technical device and to enhance the strength of the evidence for DoA-intake driving case. In this manner, confirmatory and comparative drug

identification (results from on-site OF testing devices compared with those from their laboratory analysis) was performed.

First of all, it should be noted that the sample, remaining on the collector swab of a roadside device, is the only one available for investigation. Hence, the sample preparation is crucial for research of DoA in OF on used on-site Dräger device. In this study, pre-treatment of OF collector swab with MeOH and HCl intend better extraction efficiency of the drug concerned. It is widely accepted that MeOH efficiently elutes drugs from swab whilst HCl converts them into water-soluble hydrochlorides (24, 29, 30). Therefore, the OF collector was pretreated with MeOH and HCl to increase the extraction of substances. The sample then undergoes sample preparation suitable for each analyte (corresponding to the preliminary result), which aims at more complete extraction and compatibility with the analytical technique used (24, 25).

Secondly, it should not be forgotten that some time has elapsed between roadside testing and presentation of the technical device to the laboratory during which the storage temperature is unknown (Table 4). The proper storage of specimen for toxicological analysis is of crucial importance if analytical result is to be useful in the resolving forensic cases (31). It is widespread that concentration of some DoA and/or their metabolites decreases while in storage due to degradation, temperature effects or unspecific binding to collection swab (31). Therefore, if impossible to performed confirmatory analysis immediately after receiving on-site Dräger device in the laboratory, it is stored short-term at a refrigerated temperature (4 °C) until analysis. These conditions ensure maintenance of stability of DoA in specimen (31, 32).

Based on the above, 113 on-site devices for drug testing in OF were analyzed (Table 3). The agreement between preliminary positive results and those from confirmatory analysis was highest for THC (50.00%) and AMP/MET (47.83%), when Dräger DrugTest 5000 was used, and lowest for the same DoA in case of Dräger DrugCheck 3000 roadside testing. Beside storage period and temperature, the possibility of drug recovery from collection swab, matrix effects and non-specific binding of drugs to plastics could influence on the laboratory result (31, 33). Dräger DrugTest 5000 OF collector swab was previously evaluated and it seems that it is an adequate oral fluid sample collection method (33, 34). However, there was no such evaluation for another on-site test Dräger

DrugCheck 3000 up to the moment, which concordance rate was worst in all tested compound except COC. Therefore, possible explanation could be exactly the mentioned factors.

The results of non-compliance could also be due to cross-reactivity related to medications towards any of the analytes evaluated (35-38). Totally, only in nine of all cases the driver reported the usage of medication(s), containing compound(s) such as ephedrine, ethylmorphine, pseudoephedrine, cannabidiol, ranitidine, ibuprofen, quetiapine, and diphenhydramine, that could lead to a preliminary positive result. In six of these cases, the laboratory confirmatory analysis was negative for the presence of DoA in remaining OF on collector swab. Thus, the intake of medications was not objectively proven and, accordingly, drug abuse could not be completely ruled out.

When preliminary positive results concern two or more DoA i.e., there is combined drug use, the confirmation with laboratory analysis of illicit drugs in OF, remaining on collector swab of Dräger device, reaches 31.25% for Dräger DrugTest 5000 and 12.50% for Dräger DrugCheck 3000. In such cases, the sample is processed according to the procedure, described for THC, COC, BZD and MTD, GC-MS analyzed and subsequently derivatized with BSTFA, and GC-MS re-analyzed. This process could lead to a poorer extraction and analysis of AMP, MET and OPI.

A confirmatory GC-MS procedure for analysis of DoA in OF specimen, collected from on-site Dräger devices, was presented. Data obtained showed a relatively good correlation between preliminary testing result and confirmatory GC-MS analysis. However, the storage period and storage temperature are critical to the attainment of representative results. Confirmatory analyses of used OF drug testing devices is a suitable alternative to blood testing especially when no other biological specimen (blood, urine) is submitted for toxicological analysis. Some difficulties in such confirmatory analysis should be better evaluated before implementation.

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CREATININE ANALYSIS FROM ARTIFICIAL VITREOUS HUMOR: A NEW APPROACH FOR LEGAL MEDICINE

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Vitreous Humor (VH) is defined as a sterile, colorless, and valuable forensic postmortem sample, which enables to the indication of cause of death, consumption of alcohol (acute/chronic), drug/psychoactive substances, estimation of postmortem interval (PMI), and evaluation of some pathological conditions such as Diabetes Mellitus, electrolyte imbalance. Artificial samples play a key role for comprehensive investigations once real sample is not available (1,2). To demonstrate the similarity between artificial VH (A-VH) and real VH, concordance of physicochemical properties are considered. Of these, refractive index, and density are the most important physicochemical properties that A-VH samples need to possess same as real VH (3,4). One of the VH biochemical constituents is creatinine metabolite that is an end-product of creatine metabolism. Numerous studies have conducted regarding the stability of creatinine and all studies have shown that this biomolecule is fairly stable in post-mortem period inside of the VH. It has been reported that creatinine biomolecule contributes to the both determination of PMI and diagnosis of ante-mortem renal impairments after postmortem analysis within VH (5,6,7). Accidental puncture of VH sample during autopsy may cause contamination of this sample by blood which leads to misinterpretation of results (8).

The presented study was aimed to determine A-VH's physicochemical properties and develop a new method for creatinine analysis in A-VH by both UV-spectrophotometer and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). This study also focused to demonstrate the differences of physicochemical properties and the creatinine concentration in relation to blood contamination during autopsy.

Sample Preparation

Four types of A-VH samples (Type 1, Type 2, Type 3, and Type 4) that correspond different age groups were prepared in a Phosphate-Buffered Saline (PBS) and Distilled Water (DW) by using hyaluronic acid sodium salt at a concentration of 2,20 mg/mL, 1,65 mg/mL 1,1 mg/mL, 0,55 mg/mL and agar at a concentration of 1,10 mg/mL, 1,05 mg/mL, 0,70 mg/mL, 0,35 mg/mL from Type 1 to Type 4, respectively.

Experimental Procedure

In order to demonstrate similarity between A-VH and real VH; osmolality, surface tension, density, pH, viscosity, and refractive index were measured for each artificial VH types. Furthermore, the samples were contaminated with 10% blood-PBS solution to simulate mistakes during sample collection at autopsy and subsequently, the same physicochemical measurements were performed as mentioned above.

To detect creatinine concentration in A-VH sample types, UV-spectrophotometer and LC-MS/MS method were optimized. For spectrophotometric analysis, 0,75 mg/dL and 1,0 mg/dL creatinine standard concentrations were used to spike all A-VH types. Jaffé-based spectrophotometric method was conducted and this analysis consist of two different steps; protein precipitation with 10% sodium tungstate and direct analysis. Also, all samples contaminated by blood at a rate of 10% to demonstrate the variation in concentrations of creatinine.

In LC-MS/MS analysis, creatinine d_3 was used as an internal standard (IS). All A-VH samples including 25 ng/mL IS solution were spiked with 10 ng/mL creatinine standard and *OASIS MCX* cartidges were used for solid phase extraction (SPE). To compare creatinine concentration results of contaminated and non-contaminated A-VH samples, all of them were contaminated by 10% blood-PBS solution.

Method validation

The accuracy, precision, and repeatability parameters were investigated in spectrophotometric method, in LC-MS/MS method; linearity, accuracy, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ) parameters were investigated. LOD and LOQ determinations of creatinine by LC-MS/MS system were carried out depending on signal to noise (S/N) ratios.

Statistical Analysis

Statistical analyses were carried out with Microsoft Excel 2010 student's *t-test* and p value <0,05 considered as significant.

After comparison of physicochemical properties of prepared DW and PBS A-VH samples with real VH, it was concluded that A-VH prepared with PBS was much more similar to the real VH in terms of osmolality, refractive index, density and pH as shown Figure 1. For this reason, all other studies were carried out in this way.

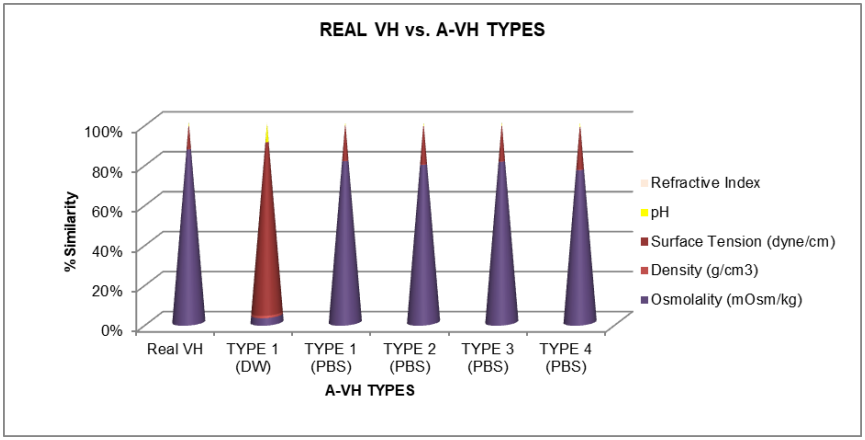

Figure 1: Comparison of physicochemical properties between real VH and A-VH samples

The mean values of 4 different types of artificial VH were ranged from 7,30-7,56 for pH; 74,97-83,88 dynes/cm for surface tension; 274,50-375,50 mOsm/kg for osmolality; 1,00750-1,00803 g/cm^3 for density; 1,33593 - 1,33616 for refractive index and 1,42-6,73 mPa.s for viscosity. Osmolality except Type 4 (*p=1,55E-05, p=8,09E-10, p=0,01, p=0,81* for Type 1-4 respectively;95% CI), pH (all of them *p=0,01*;95% CI) and viscosity (all of *p=0,01*;95% CI) p-values of A-VH that were contaminated by blood were significant, but density (p=0,74,p=0,07,p=0,12,p=0,42 for Type 1-4 respectively;95% CI), surface tension except Type 2 (p=0,56,p=0,01,p=0,1,p=0,81;95% CI) and refractive index (p=0,34, p=0,42, p=0,46 p=0,09; 95% CI) values were not significant.

The spectrophotometric results of 0,75 mg/dL and 1 mg/dL

creatinine spiked and protein precipitated (PP) A-VH found 0,60-0,84 mg/dL, 0,85-1,09 mg/dL, respectively. The direct analysis results were 0.60-0.72 mg/dL and 0.90-0.93 mg/dL, respectively. It was demonstrated that there was a significant decreasing in the creatinine values in blood-contaminated A-VH samples for both PP and directly analyze method. It was observed that direct analysis of creatinine by spectrophotometric method is more accurate with ≤5.89% relative standard deviation (%RSD) for the analysis of creatinine and also, found to be more appropriate and less time consuming.

The linear range of new developed LC-MS/MS method was 0,005-100 ng/mL and r^2 was 0,999. The total run time was 5,5 minutes the retention time of creatinine was 1,0 minutes, whereas creatinine d_3 was 0,989 minutes. In this study, LOD of creatinine was 0,005 ng/mL, while the LOQ was 0,02 ng/mL. In the process of SPE, it was concluded that the OASIS MCX cartridge provided a repeatable, accurate and more precise results for extraction with ≤1,45 RSD%.

The creatinine concentration –for 10 ng/mL spiked A-VH samples was found between 8,57-9,01 ng/mL with 85,70-90,10 Recovery% while blood-contaminated samples was found to be 6,20 ng/mL with 62,00 Recovery%. It was revealed that blood-contamination caused a significant *(p=0,01)* decrease in creatinine concentration.

The osmolality (except Type 4), viscosity and pH values of A-VH samples significantly have differed from those of non-contaminated A-VH values, whereas surface tension (except Type 2), refractive index, and density values have changed insignificantly. We have measured relative viscosity of A-VH, however, previous studies generally performed with dynamic viscosity. As a limitation, we couldn't compare the similarity of viscosity values between real VH and A-VH. On the other hand, surface tension values of A-VH were greater than real VH surface tension measurements. The reason could be attributed to the limited chemical composition of A-VH (9). After blood-contamination of all A-VH samples, osmolality (except Type 4), pH, and viscosity values were significantly changed. The osmolality value is influenced by the amount of constituents that matrix contains (10), for this reason, osmolality values of the blood-contaminated A-VH types were found significantly different from non-contaminated ones. Due to the ingredients of blood, pH values

have changed significantly after blood-contamination. Because of the increases of molecular interaction/forces, A-VH viscosity values have significantly changed with contamination.

It was concluded that UV-spectrophotometer method with direct analysis is more reliable than with protein precipitation. By using LC-MS/MS, it has shown that this sophisticate method is more accurate, sensitive, and advantageous. Lastly, contaminated A-VH samples have exhibited insignificant changes as compared LC-MS/MS method in particular accuracy and recovery results.

Firstly, it was indicated that PBS based A-VH was the most similar to the real VH in terms of osmolality, density, refractive index, and pH values. Secondly, we have demonstrated the physicochemical varieties between contaminated and non-contaminated A-VH samples. On the other hand, age-dependent physicochemical measurements have carried out for the first time with this study. Also, two different methods were validated for creatinine analysis in A-VH samples by using UV-spectrophotometer and LC-MS/MS. This study would be a pioneer for uses of A-VH sample in forensic science investigations. This study would shed light on diagnosis of antemortem kidney impairments by analyzing creatinine in VH with LC-MS/MS for further studies. Moreover, we have demonstrated the differences of creatinine values in relation to blood contamination. Hence, blood-contaminated samples would some alterations of creatinine-based analysis; therefore, new algorithms that contain contamination factor would be established.

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COMPARISON OF DIGITAL EVIDENCE OBTAINED BY DIFFERENT METHODS FROM IOS PHONES SEIZED FROM CRIME SCENE

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Thanks to the rapid development of technology, communication has become very fast. With the introduction of computers, mobile phones and the internet into our lives, we started to carry out many of our activities through these devices. People communicate, play games or even socialize with their friends, work from their homes, buy everything they want from online markets, boutiques etc. also they can make every banking transaction through online with the help of these technologies. People can fulfill all of these daily tasks and basic needs such as communication with their mobile devices which they carry all the time.

People use mobile devices to communicate or interact with each other. In addition to performing daily activities, people can also use these devices while committing various crimes. These devices can also help criminals to communicate with each other, share or publish illegal files, documents or violate privacy of others. Any crimes related with these devices can contain valuable information (evidence).

Most of the mobile device users communicate and interact with each other through instant messaging apps. One of these messaging app Discord is an application that allows text, image, video, and audio communication using VoIP. Discord is built around a network of private and semi-private groups, known as "servers," which are created by mostly anonymous users. And this anonymity attracts criminals to use this app.

There are few mobile device manufacturers in the world and they are committing to security and privacy to their customers. Although this security measures are designed to protect users from crime and criminals, they also complicate law enforcement's efforts when it comes to investigating a crime.

iPhone, one of the most popular mobile devices, uses the iOS operating system developed by Apple. According to Apple, there

were 1.65 billion active iPhone users worldwide in January 2021. (1) Apple, like other manufacturers, takes the security of these phones seriously, so they take advanced security measures. iPhone's security restrictions limit the capabilities of evidence collection of law enforcement officials.

Mobile forensics, which is a branch of digital forensics, evidence can be obtained from live memory analysis or forensic image, as well as by listening to network traffic. These three methods have advantages and disadvantages. Digital forensics experts can mostly use forensic image acquisition rather than other methods due to security restrictions of mobile devices. In order to gather the most evidence, including deleted data, physical image acquisition should be performed. However, forensic software used for forensic image acquisition can sometimes be encountered in cases where new version mobile devices do not support (cannot pass security measures), physical images cannot be obtained and less evidence can be accessed.

Our study aims to compare the evidence obtained with these three methods used in the field of mobile forensics and what the evidence can be obtained by using these three methods on phones using the IOS operating system.

For performing three forensic methods, two separate iPhone used in our research and one of them was jailbroken to bypass security measures (to dump live memory for memory forensics) and the other one left as it was. (Table 1) And then we installed Discord instant messaging app to interact between two phones. We texted, shared documents, picture and other different types of files.

To capture network traffic, we used Burpsuite community edition and prepared devices;

•Settings > Wi-Fi > Network Name (SSID) > Set up Proxy > Manual

•Server > IP address of the forensic workstation (computer) and port number we defined on Burpsuite (by default 8080)

•On a web browser opened http://burpsuite page and click "CA certificate" button and downloaded SSL certificate

•Downloaded certificate set up on Settings > General > Profile Downloaded

•Profile activated as "trust root certificates" under Settings > General > About > Certificate Trust Settings (2)

To dump live memory, we used Fridump.py python script (3) which is an open-source tool for dumping uses the Frida framework to dump accessible memory addresses from any platform supported. It can be used from a Windows, Linux or Mac OS X system to dump the memory of an iOS, Android or Windows application.

On a forensic workstation computer to installed iTunes and we downloaded Fridump from github web page and run it:

•https://github.com/Nightbringer21/fridump

On the jailbroken iPhone (connected to forensic workstation via USB cable)

•Start Cydia and add Frida's repository by going to Manage -> Sources -> Edit -> Add and enter https://build.frida.re.

On forensic workstation, command line interface or Powershell can be used to list the running processes on iPhone to dump the memory (we chose the Discord app)

•frida-ps -U (list the running processes)

•com.hammerandchisel.discord is the identifier of Discord app

Dumped the memory of the app with the following command

•python fridump.py -U -s com.hammerandchisel.discord

For acquisition of forensic image, we used UFED 4PC software and UFED Physical Analyzer for analyze the image. According to UFED instructions we prepared the device, the preparation is changes with the device model we used iPhone 6

•Placed the device in recovery mode. (The Apple iTunes logo should appear.)

•Pressed the "Power" button for three seconds.

•After three seconds simultaneously holded both the Power and "Home" buttons down for an additional 10 seconds.

•Released the Power button while holding the home button for an additional five seconds.

•Cellebrite UFED "Continue" should now be enabled. (4)

Specifications	iPhone	iPhone Jailbreak
Brand	Apple	Apple
Model	Iphone 6	Iphone 6
Operating System	IOS	IOS
Memory	16 GB	32 GB
Version	12.5.1	12.5.1

Table 1: Specifications of test phones

Specs	Acqusition	Analysis	Packet Capture	Memory Dump
Brand	UFED	UFED	Burpsuite	Fridump.py
Model	4 PC	Physical Analyzer	Community Edition	N/A
Version	7.44.080	7.44.13	V2021.8.3	0.1

Table 2: Tools used

Before starting process, we connected two iPhones to separate forensic workstations (Operating System Windows 10) via USB cables.

Set forensic workstation computer as a proxy and start to capture network traffic through Burpsuite.

Test cases (interactions between two phones)

•We set up a server through "jailbreakediphone" user

•Chat through general chat room (channel) for the "jailbreakediphone"

•Shared documents and pictures

•We called other party

While interacting between to phones traffic captured through Burpsuite.

After these interactions; we dumped memory of the phones before shut down using "fridump.py"python script, after shut down physical acquisition is performed with UFED 4 PC.

UFED Physical Analyzer is used to analyze the forensic image and we searched for these evidences.

Evidence Type	iPhone	iPhone Jailbreak
IP addresses	78.188.98.XXX	46.106.27.XXX
Email addresses	noniosjb@mail.com.tr	gameriosjb403@mail.com.tr
Chat messages	"Noniosjb silinecek mesajdan sonraki"	"Silinecek yanit304"
Shared Files (Pic, Pdf)	20210509_211719.jpg TOURISM_IN_PARIS.pdf	Collesum.pdf
Call	Voice_connection_success	Voice_connection_success

Table 3: Evidence Types

This section presents the analysis results for Discord app on two iPhone (jailbroken and non-jailbroken). We set up a controlled environment for populating data to be analyzed on two separate iPhone with different security level. The jailbroken iPhone does not have security restrictions as much as non-jailbroken device. The evidence availability can change due to the security restriction of these phones. In our study, we investigated different digital evidence gathering methods of mobile forensics and we attempted to determine which method can obtain more evidence and how these

methods contribute to the investigations of digital forensic experts.

As we mentioned before there are some prerequisites for performing all three forensic methods. When we try to memory dump, we should connect it through USB and put a small python script to memory to read/dump it. For network analysis we prepared the device send network packets through forensic workstation (set up as a proxy server) and implemented root certificate of Burpsuite to decrypt the traffic. After these live captures when acquisition a forensic image, we put the phone in recovery mode. They are all need to interfere somehow with phone. In real life investigator may not perform live memory dump because of the security restrictions or can't run a python script like fridump. Preparation of the devices for network capture or extraction of memory dump is quite harder than data extraction from physical image of the iPhone.

IP address is the most valuable evidence to detect and track the location of the criminals. But We could not detect the IP address of the other iPhone which was simulating the criminal. The reason would be any application communicates through the app servers and does not connect directly peer to peer.

E-mail addresses, we created used for setting up user accounts on Discord app. In the analysis phase these e-mail addresses detected with all three methods. In forensic image cache.db When we analysed the findings observed that the email addresses are found with string word "email" and if we search this string as a default keyword, we can detect email address (Table 4)

When we chat through discord app, we captured API (Application Programming Interface) calls (/api/v9/channels/{channel_id}/ messages) which send and receive the messages associated with the channel identifier number. The memory dump and forensic image was searched for keywords and we found these chat messages also from these evidence sources. To detect email addresses, phones' security measures does not affect and they are not necessarily need to be jailbroken. (Table 4)

Voice call through Discord app is also detected as an API call (/api/v9/science) on network analysis. When analyzing forensic image, it was observed that "Cache.db" database file located under this file path also contains chat messages and voice calls.

DarArchive/root/private/var/mobile/Containers/Data/Application/5E16CEA1-328B-4E56-9D8C-1953C5338EA8/Library/Caches/com.hammerandchisel.discord/

The memory dump only could be extracted from the jailbroken iPhone and from non-jailbroken device it could not be. We detected e-mail addresses, chat messages and shared file names not the entire file on memory dump.

Evidence	Non-Jailbroken iPhone			Jailbroken iPhone		
	Network	Memory	Physical Image	Network	Memory	Physical Image
IP Address	No	N/A	No	No	No	No
E-mail address	Yes	N/A	Yes	Yes	Yes	Yes
Chat Messages	Yes	N/A	Yes	Yes	Yes	Yes
Shared Files	Yes	N/A	Yes	Yes	Partially Yes	Yes
Call	Yes	N/A	Yes	Yes	No	Yes

Table 4: Evidence Results

The Discord application, which was developed to provide communication between gamers, is also used by criminals to carry out illegal activities due to its various anonymity features. (5) In this paper (6), researchers carry out some analysis on computers with different operating system. This research is performed on computers and does not cover the mobile forensic approach.

Our main goal was to perform three different evidence gathering method on iPhone devices. And there are multiple researches about

IOS/iPhone forensics. In this research (7) Barrios et.al explained acquisition methods of IOS devices and challenges performing acquisition. In this research (8) researchers emphasized three different analysis methods. Bhatt et.al (9) performed network analysis on iPhone with 20 different social media apps, and focus on privacy issues.

Currently, in literature search we determined that most researches and analysis of mobile forensics are focused on static data on forensic images on non-volatile memory. But if some conditions are present or these conditions can be created, evidence can be collected over the network and on the live memory dump. There are some preparation steps if a forensic experts want to gather evidence on the network or if it is necessary to take a memory dump. We simulated these conditions and determined if any other kind of evidence could be obtained with the other methods.

Digital forensic experts must have the necessary equipment and competence to intervene in digital evidence when they arrive at the crime scene. If some prerequisites are present, it will be possible to detect very valuable information by listening on the network or extracting the memory dump of the phones.

In our study we concluded that:

•Physical Image is the less interfering method

•Memory dump can be only performed on jailbroken devices

•Network capture and memory dump needs some preconditions

•Sometimes crime scene experts can seize jailbroken devices from crime scene and they can perform other two forensic methods.

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EXAMINATION OF TIME DEPENDENT VARIATIONS IN SIGNATURES

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Signatures generally are illegible scribble, pictogram or design which consist of handwriting characters in the name and surname of a person(1) and reflects the personal writing characteristics and habits (2).

Turkish Surname Law Number 2525, Art. 2 indicates that "in utterance and correspondence; the first name is used in front, surname at the end of the signature". Although this law states that the signature should be made with handwritten characters of the name and surname, there are differences in terms of legislation and implementation in terms of how the signature should be (3). Since this law cannot be used effectively, it is seen that signatures are mostly in the form of symbols in practice.

According to Huber and Hedrick, signatures evolve through in the stages that are learning, puberty, maturity, and senility stages in the course of a person's life. The most consistent shape of the signatures appears in the maturity stage, except the person does not have any neuromuscular system or any health problems or medication treatment (4). The study of (5) shows that education degree, frequency of signature usage and aging play an important role in the signature changes over time. During the senility writing quality, pen control and fluency in signature are degenerate progressively. Advanced age also affects handwriting speed and causes differentiation on the signature construction (6).

The study of Caligiuri and Linton indicates the effect of age on the variation in signature and according to this study, young people construct with more pressure when they make upstrokes in the signature than the elders (7).

Some individuals just use signatures consisting of simple drawings and signs called "stylistic signature" instead of writing name and surname depending on educational degree (8,9). The smooth, continuous, rapid and rhythmic signature created by a qualified person can stand out with its artistic appearance. In contrast to this; hesitant, interrupted, laborious and slowly executed signatures may

have been produced by less skilled people (10).

In addition, signature construction can be affected by some factors such as writing position, writing surface, changes in health of the writer, physical and mental conditions of the writer, influence of medications, drugs or alcohol (1,11).

Signing position (including stance) is an important factor that can cause variation in signature. Signing while sitting may have a different slant or size in the signature versus while signing in standing position or in a hospital bed without hand or arm support. In case of a rush, people may attempt to sign on their lap or knee. Signing position often causes altering the signature. Taking signatures in a standing position without support causes substantial changes in signature. Moreover, when signing in small spaces or boxes on documents, some characteristics of signature are changed in execution (12).

Paper containing flax or backed with pieces of wood fiber can cause distortion of the lines. Signing on floors that are uneven and do not contain support (poor quality pad or paper leaning against the wall) may cause the signatures to be misinterpreted and identified (13,14) .

The structure of the pen, which is a writing tool, also affects the speed and fluency of the signature. A fluid ink pen makes the signature look more fluent, while the ballpoint pen does not show this fluency as it sometimes creates difficulties for the signer. A signer often has to complete the signature by going over the lines repeatedly. This situation causes the original lines of the signature to be mixed with the lines originating from the pen (13).

Changes in health may affect the fluency, rather than the designs of signature. The changes can be expected to be temporary, however, and writing facilities will likely return with the recovery of health. The deterioration like motor impairments (e.g. Parkinson's disease) of health may cause a loss of control and more erratic movements. Fractures of the hand and arm requiring restraints on mobility that inhibit the grasp of the instrument or the movement of the pen will alter the signature. Furthermore, schizophrenics or people with multiple personalities can exhibit major changes in their writings and signatures on different situations corresponding to the mental state at the time (7).

It is known that physical and mental conditions affect signatures. The effect of stress, which is an important factor on signature, is

divided into two as physical and emotional. Especially in the presence of excessive mental or emotional stress, the person may be under the influence of this stress while signing. In a situation where a person is extremely relaxed, there is carelessness or slackness in his/her signature In the case of fatigue that causes physical stress, when the person signs several documents or signs several signatures in one sitting, changes in the signature can create problems in terms of identification (7,13).

Some characteristics of the signature may change under the influence of drugs and alcohol. Long-term addicts may show less variability in their signature when signing under the influence than those who are non-addicts and less regular drinkers. Some medications cause tremor and disturbance of motor movements while other medications associated with neuro-muscular diseases may decrease tremor and disturbance of motor movements. In such cases, stroke quality of signatures may be varied positively or negatively (15).

In some cases, there are accidental occurrences in writing signatures that may have no explanation like unusual forms or movements, breaks in the writing line, or even the doubling of some letters or parts of letters (16).

A minority of people can use both hands with the ability to write with either hand with almost equal dexterity. In most respects, the written products are similar. Muscular coordination of the two hands and arms may not be precisely the same, however, and differences in fluency and some movements may be noted (13,17).

Concentration on the writing act causes the action to become more deliberate and slower. Most writers have occasions when their writing degenerates to a scrawl or scribble due to haste, carelessness, or particularly poor writing circumstances (13).

It is possible for variation to develop due to some additional conditions. Some standards are the product of time, the signature itself, and the environmental conditions (13).

In this study, we aimed to define if some characteristics of signature are changed or not when the signing action is fast or when the signing action takes place by the end of the working day.

In this study, the sample group consists of 10 participants between the ages of 25-55. 6 (%60) of the participants are female and 4 (%40) are male. Educational status varies between high school and doctoral graduates.

The signatures of the participants on the timesheets that are collected for one-year period were evaluated based on the time intervals selected by the researchers at Questioned Document Laboratory of Istanbul University-Cerrahpasa, Institute of Forensic Sciences and Legal Medicine.

First, seventh, fifteenth, thirtieth, ninetieth, one hundred and eightieth and three hundred and sixtieth day were time intervals of collecting samples.

The signatures were examined and categorized independently by three different document examiners unaware of each other. If their decisions were compatible with each other, the result was accepted. If there was a difference between them, they discussed and tried to reach a common result to avoid inter-observer error.

The participants signed the time sheet, which is in the file with 4 papers underneath. Participants signed using ballpoints which are the same brand (Pensan, 1 mm) but in two different colors (black and blue) during a one-year period.

In cases where the participants could not sign because they were not in the institution, the time sheets from the previous five days and/or the next five days were examined.

Hypothesis:

H 1. As the act of signing becomes routine throughout the year, people are expected to sign faster and with less pressure, therefore signatures are expected to be signed with less characteristic and diacritics.

H 2. Due to the fact that the second signatures are signed by the end of the working day, people are expected to sign faster with less pressure and change their signature characteristics in a hurry to leave the institution.

The definitions of the variables examined in the study are listed below:

Complexity: Alterations in quality of signature characters and difficulty in construction within a year.

Pressure: Alterations of the pressure level within a year.

Speed: Alterations of the stroke line speed within a year.

Deviation angle from the baseline: Alteration in the angle of deviation of signatures from baseline within a year.

Aspect Ratio: Alterations in x/y dimension ratio of the signatures within a year.

Special Marks: Alterations in the presence and position of

diacritics in signatures within a year.

We observed that the participants of this study have been using letters that consist of characters in their name and surname like scribbles as a signature. None of the differences in signatures was based on being ambidextrous, because all participants were actively using only one hand.

The participants were more careless during signing the time sheet compared to signing significant documents like mortgages, financial documents and real estate transactions.

Analyzing results of the signatures which were collected in different time intervals (1st, 7th, 15th, 30th, 90th, 180th and 360th days) are shown in Table 1.

Sample	Complexity	Pressure	Speed	D. A. from B*	Aspect Ratio	Special Mark
S 1	Low to High	Same	Same	Same	Increased	Placement differences
S 2	Same	Increased	Decreased	Same	Increased	N/A
S 3	Same	Increased	Decreased	Decreased	Same	Omitted
S 4	Same	Same	Same	Same	Same	N/A
S 5	Same	Same	Same	Same	Same	Same placem
S 6	Same	Increased	Decreased	Same	Same	N/A
S 7	High to Low	Same	Same	Same	Decreased	Placement differences
S 8	High to Low	Same	Same	Same	Increased	N/A
S 9	Same	Increased	Decreased	Same	Decreased	N/A
S 10	Same	Decreased	Increased	Same	Same	N/A

Table 1: Variations in the signature of the participants over a year.
* Deviation angle from the baseline

According to examination results, the signature speed of 4 (%40) participants has decreased while signature pressure has increased or both have remained constant for 5 (%50) participants. While 7 (%70) participants kept their signature complexity and 9 (%90)

participants-maintained deviation angle from the baseline of the signatures. 5 (%50) participants kept their aspect ratio the same. When the change of the second signature on the same day is also examined, it was observed that the second signatures of 4 (%40) participants had more pressure whereas 2 (%20) participants had less pressure; 3 (%30) participants had a tendency to exaggerate the initial stroke; 4 (%40) participants had a tendency to decrease the number of mid characters; 6 (%60) participants had changes in their last figure and 4 (%40) participants had similar characters in comparison with first signatures.

One of the participants has a signature with a high degree of complexity. The intricacy of movements and the complexity of patterns are important elements in signature analysis. However, the lack of methods used to measure complexity and intricacy in the literature is an important problem (Huber and Headrick, 1999, pp.33). Analyzing this signature was a challenge.

In some signatures, it is seen that the angle of the signature is different in a year-period, but this situation may stem from the different placement of the time sheet on the desk.

While no significant change was observed among the signatures of the individuals within a year, it was observed that there were significant changes in some characteristics of the signatures that were signed during the day (morning and evening).

All signatures were stylistic as parallel to Hilton's findings, but participants with short signatures consisting of fairly simple symbols and shapes were not included in this study (8).

The contrary of Caligiuri and Linton 's study which indicates the young people construct with more pressure when they make upstrokes in the signature than the elders; we conducted that the pressure is not related with age but it can be changed due to signing in restricted time (7).

Signing under the influence of stress causes signatures to lose some characteristics (7,13). In this study, we also observed that the stress caused by reasons such as trying to catch the shuttle and trying to get home at the end of the day, affects the signature.

According to Dixon, Kurzman, and Friesen's study, the signature changes over time. However, as limitations of this preliminary study, one year period signature collecting and limited number of participants prevented the examination of the signature changes for a long time (6).

Contrary to what was claimed in *Hypothesis 1*, it was observed that the pressure remained the same or increased, however, a detailed examination could not be carried out on the subject of diacritics. If a larger sample is studied, the change in diacritic can be observed better.

Hypothesis 2 is unconfirmed regarding pressure and speed; however, it was partially confirmed by the decrease in the mid characters and the last figure changes in the signatures of the majority of the participants.

The number and non-uniformity of samples were the restrictions of this study. Studies that are more comprehensive can be carried out by expanding the sample group and evaluating many factors such as age, gender and occupation together.

This study is a preliminary study carried out with a limited number of participants in a limited period. The scope of the study can be expanded by increasing the number of participants and collecting signatures over a longer period.

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THE IMPORTANCE OF PHOSPHATIDYLETHANOL (PETH) ALCOHOL BIOMARKER IN FORENSIC SCIENCES - A BRIEF REVIEW

Fatma Beyza Kula, Dilek Salkım İşlek, Emel Hülya Yükseloğlu

Alcohol is an easily accessible legal drug and used in social environments. According to a report published by Traffic Safety Data and Analysis Group, fatalities in traffic could be from 10% to 30% worldwide as a consequence of driving under the influence of alcohol (1). In addition, severity of a traffic accident could be more serious if a person drives after alcohol intake (2). Considering the importance of alcohol for forensic sciences, determining the amount of alcohol consumed is essential. Ethanol can be detected from blood, urine, and breath among which urine has the longest detection time (3).

The detection time of ethanol is short; thus, using the metabolites of ethanol could be more helpful to guide the forensic cases accurately. There are indirect and direct alcohol biomarkers to identify alcohol consumption. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), mean corpuscular volume (MCV) and carbohydrate deficient transferrin (CDT%) are indirect biomarkers of ethanol and provide information about indirect response of body after the ethanol consumption (4). CDT% is the most sensitive indirect biomarker for chronic alcohol consumption with sensitivity up to 90% and specificity up to 95% (5). The indirect biomarkers generally used for supportive evidence of alcohol use due to the fact that indirect biomarkers could be affected by diseases and drug use (4, 6).

In interpreting alcohol intake more accurately, direct biomarkers, which are ethyl glucuronide (EtG), ethly sulfate (EtS), fatty acid ethyl esters (FAEE) and phosphatidylethanol (PEth), are relied upon due to their power of being direct ethanol metabolites (4, 6). EtG can be detected from both urine and blood for routine use but the concentration of EtG in urine is higher than blood (7). EtG from urine could be detected between 15 and 25 hours if 0.5 g/kg of ethanol is consumed (8). However, urinary creatinine levels should be taken into account due to the fact that urine dilution could affect

the concentration of EtG in urine (9). Similar to EtG, EtS could also be detected from urine and influenced by urinary creatinine levels (4). Another direct alcohol biomarker, FAEE, could be found in blood, hair, sebum, meconium etc. (10); however, FAEE is generally used to detect heavy alcohol use from hair (11). In recent years, PEth has caught attention due to its long half-life in blood (12, 13), not giving false-positive results (14-16). The aim of this review is to present a brief overview about the potential use and limitations of PEth alcohol biomarker in forensic sciences.

Studies conducted between 2010 and 2021 were searched on Google Scholar and Pubmed to summarize the clinical use of PEth biomarker for social, moderate, and chronic drinkers. In this review, studies on measuring blood-PEth were included and studies related to liver diseases, HIV and etc. were excluded to better understand the routine use of PEth in forensic sciences.

Phosphatidylethanol (PEth)

One of the most interesting phase II metabolites of ethanol is a different type of phospholipids called phosphatidylethanol. PEth was first discovered in some organs of mouse that was treated with ethanol for 3 weeks (17). However, PEth had not been established as a potential biomarker for ethanol consumption until the study just mentioned was conducted in an alcoholic detoxification program (18). PEth is not a single molecule but is made up of 48 different phospholipids discovered in cell membranes (19). The importance of the biomarker is that it is only formed in the outer cell membrane under the presence of ethanol which distinguishes PEth from other biomarkers. Outer cell membranes are composed of phosphatidylcholine (PC), and under normal conditions with the help of phospholipase D (PLD) enzyme, PC is converted to phosphatidic acid (PA) and choline molecule (4, 12, 20). Interestingly, PLD enzyme has more affinity to ethanol than water; thus, PEth is formed only if there is alcohol consumption (21, 22).

Homologues of PEth are defined as the ratios found in the body after alcohol consumption and the amount of PEth could vary in individuals (23). The most important ones are reported to be PEth 16:0/18:1, PEth 16:0/18:2 and PEth 16:0/20:4 by 37%, 25% and 13% ratios, respectively (13,19). The notation of PEth X:Y/Z:W can be interpreted as follows: X and Z are the number of carbon atoms in the chain Y and W are the number of double bonds in that carbon chain (13).

PEth is a promising alcohol biomarker because it is not affected by gender (24, 25), age, liver disease (26), or diabetes mellitus (27).

Unlike the indirect biomarkers detected in the body after some period of time, PEth has high specificity and sensitivity which makes it more reliable than other biomarkers (14). It has also a long window of detection time up to 14 days for social drinkers and 28 days for chronic alcohol users with a half-life found between 4-7 days (24, 28).

In determining the concentration of PEth, thin layer chromatography (TLC) method was used at first but it was not suitable for quantitative analysis (29) so, high performance liquid chromatography evaporative light-scattering detection (HPLC-ELSD) method was introduced (30). Later, capillary electrophoresis coupled with UV (31) was shown to be a novel method to detect PEth. Increasing the sensitivity for PEth measurement, HPLC coupled with mass spectrometry (MS) was used for routine use (13).

There are important mechanisms to be discovered for the formation and elimination of PEth for more efficient use in forensic sciences. A selected group of studies about PEth from 2010 to 2021 to understand the mechanism and the importance of PEth are summarized in Table 1 for social and moderate drinkers and Table 2 for chronic drinkers.

Studied Biomarkers	Studied Matrices	Study Groups	Study Design	Results	Reference
PEth 16:0/18:1 CDT GGT	Whole blood	Healthy social-drinkers	Studying volunteers with 3 weeks of abstinence and requesting them to consume alcohol for 5 consecutive days to reach BAC with 1 g/kg by blood collection of 20 days	PEth can be detected as long as ethanol is present in the body and there is a concentration and half-time difference between alcoholics' PEth values versus social drinkers': alcoholics have higher PEth values and shorter half-lives of PEth	(12)
PEth 16:0/18:1 CDT ALT AST GGT MCV	Whole blood Serum	44 healthy subjects	Subjects were randomly assigned to consume 150 mL of red wine (moderate drinking) every day for 3 months and were asked to abstain from alcohol for 3 months	PEth could be beneficial in distinguishing abstention from moderate alcohol consumption, and PEth was the only biomarker to detect moderate alcohol use. PEth showed a correlation with participants' reported habitual alcohol consumption. In addition, there were significant correlations between PEth and CDT and AST and ALT	(32)
PEth 16:0/18:1 PEth 16:0/18:2 BAC BrAC	Whole blood	27 healthy participants	After one week of abstinence monitored by TAC monitor, volunteers were asked to consume 0.25 g/kg or 0.5 g/kg of alcohol and PEth levels were measured for 14 days	0.25 g/kg and 0.5 g/kg single dose of ethanol increased both BrAC and PEth levels. Half-lives of combined PEth was 4.6 days due to difference of half-lives between PEth 16:0/18:1 and PEth 16:0/18:2 and PEth homologues could be used for time estimate of the last drink	(28)
PEth 16:0/18:1 PEth 16:0/18:2 EtG EtS BAC	Whole blood Urine Hair	16 healthy volunteers	Single-dose of alcohol was prepared to reach 1 g/kg BAC levels for two weeks to determine PEth levels	PEth 16:0/18:2 was detected at a lower concentration than PEth 16:0/18:1 and the latter homologue was found between 3-12 days for social drinkers	(33)
PEth 16:0/18:1 PEth 16:0/18:2	Whole blood	56 light and heavy drinkers	Deciding heavy and light drinkers by using TLFB interview, participants were asked to abstain from alcohol for a week and abstinence of alcohol was monitored by TAC monitor. Participants	0.4 g/kg and 0.8 g/kg single dose of ethanol increased PEth levels proportionally and PEth 16:0/18:2 synthesized at a greater rate than PEth 16:0/18:1; the mean half-life of PEth	(25, 34)

Table 1: PEth studies on Social and Moderate Drinkers

Studied Biomarkers	Studied Matrices	Study Groups	Study Design	Results	References
MCV ALT AST GGT CDT FAEE EtG EtS PEth	Serum Whole Blood Hair	DUI participants	Examining the indirect and direct biomarkers, studying AUDIT, TLFB, DRINC, TRI as psychometric evaluation The duration time of the study:8 months	PEth was the best biomarker to correlate with BAC and psychometric tests compared to its counterparts	(35)
PEth (14 homologues)	Whole blood	DUI cases	Sampling blood from withdrawal patients for a period of time with detailed AUDIT and one time sampling blood from social drinkers with drinking behaviors questionnaire	Among 14 homologues, PEth 16:0/18:1 was predominant for both alcoholic and social drinkers; PEth 16:0/18:2 homologue was used for determining the time of abstinence	(36)
PEth 16:0/18:1 PEth 16:0/18:2 BAC	Whole blood	DUI cases	Determining cut-off values for PEth 16:0/18:1 and 16:0/18:2 by examining BAC levels of DUI cases with prolonged excessive drinking	Cases with BAC ≥ 1.6% levels were accepted in the category of excessive drinking and the cut-off levels were determined as PEth 16:0/18:1 ≥ 700 ng/mL and PEth 16:0/18:2 ≥ 300 ng/mL	(37)
PEth 16:0/18:1	Whole blood	Alcohol-dependent patients in a clinical trial to reduce alcohol consumption	Patients' level of alcohol biomarkers were examined, and self-report of AUDIT and AUDIT-C tests were also studied	Both PEth and CDT were positively correlated with self-report with PEth being superior than CDT for higher sensitivity; moreover, no difference between sexes was found compared to PEth half-lives	(38)
PEth 16:0/18:1 PEth 16:0/18:2	Whole blood	DUI cases	DUI cases with BAC levels ranging from 0.0‰ to 3.12‰ were examined to differentiate between moderate and	PEth risen up the chance of determining excessive alcohol consumption by 31.6% compared to BAC threshold of 1.6‰. For	(39)

Table 2: PEth studies on Chronic Drinkers

Detection of alcohol consumption in traffic could be difficult and PEth might be a helpful biomarker for it when it is used with other biomarkers. In a study on alcohol ignition interlock program, drivers were examined according to their blood alcohol concentration (BAC), other biomarkers and psychometric tests; and even though BAC and other biomarkers failed to detect alcohol intake, PEth showed higher accuracy in detecting the drivers' alcohol consumption (40).

In determining the amount of alcohol consumption with PEth, important factors such as PC as a precursor, PLD enzyme polymorphisms and activity, PEth elimination rates need to be examined due to the fact that the concentrations of PEth 16:0/18:1 and PEth 16:0/18:2 vary between subjects (25). In an in-vitro-study, PEth formation and its relation to PC composition in the organs of a pig, a calf, and a goat spiked with different alcohol concentrations were examined. As a result of this study, researchers found that there is a relation between PC substrate availability and PEth formation in the organs of a pig, a calf, and a goat. (41). Likewise, in a 2019 in-vitro study, PC and PEth concentrations were correlated with each other, and the formation rate of PEth 16:0/18:1 could be affected from the amount of alcohol intake and PC 16:0/18:1 concentration level (27). Similarly, PLD is a complicated enzyme and activity of the enzyme might be related to the amount of ethanol consumption because of it is saturated even with a low dose of alcohol (25).

PEth is generally examined from blood, and if ethanol is present in blood after sample collection, PLD enzyme would convert PC to PEth which could cause the interpretation of the alcohol intake inaccurately (42). For analyzing PEth, most commonly used method is collecting venous blood samples. Since venous blood sampling is invasive, requires medical staff, and transportation and storage of the samples are challenging, and post-PEth formation might occur due to presence of ethanol in the samples, it is seen as a disadvantageous method (43, 44). Even though concentration of PEth is stable when stored at −80°C, PEth may be formed after sampling and transporting the samples to the laboratory. Eliminating the possible risk of post-PEth formation, PLD enzyme inhibitor is added to blood tubes (42).

Alternative methods of collecting the blood samples and eliminating the enzyme activity of PLD are essential. One of the methods called dried blood spots (DBS), a non-invasive method,

was first introduced by Ivar Bang and later used to diagnose phenylketonuria by drawing blood from newborn babies and dripping it on Guthrie cards (45,46). One of the main advantages of DBS is that it maintains the stability of the biological material to be analyzed for a certain time even at room temperature at the same time collecting, storing and transporting the samples are easy (47). Drying period of DBS method could vary depending on the humidity of the environment, the type of the cards and the sample to be dried; however, the average time for drying period was observed to be between 2 and 3 hours (48). The individual's hematocrit value is also important in the absorption and distribution of the sample on the card, and a pattern can be created on the card accordingly (49). In addition, heterogeneous of the blood on the card and the difficulty of repeated or additional analyses could be counted as some of the reasons why DBS method is not preferred over venous blood (50).

When DBS method and whole blood are used in determining the concentration of PEth, it has been realized that there is a good agreement between two methods (51); besides, capillary DBS has showed greater stability for PEth measurement than venous blood and venous DBS since it is not affected by hematocrit value, punch localization, or spot volume (52).

Measuring the amount and time of alcohol intake is a challenging task in forensic sciences, and one of the interesting alcohol biomarkers called PEth could be used routinely due to its high sensitivity and accuracy. Unlike other routinely used biomarkers, PEth can only be produced under the presence of ethanol and PEth has longer half-lives between 4 and 12 days. Even though most PEth 16:0/18:1 and PEth 16:0/18:2 homologues are predominant in the body, other homologues of PEth could provide better understanding of the time and amount of alcohol intake (34, 36). Further kinetic studies should be conducted such as PLD enzyme activity, PEth precursors and PEth elimination rates for the interpretation of PEth homologues correctly. While interpreting alcohol intake, PEth should be combined with other biomarkers such as CDT%, EtG which could yield better results and help to guide justice system accurately. In measuring PEth in traffic sites and preventing post-PEth formation, DBS method could be preferred over collecting venous blood.

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NANOSCIENCE AND FORENSIC GENETICS

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The technology that based on the understanding and control of physical, chemical and biological phenomenon in nanometer scale, developing and producing of nanometer scaled functional materials, tools and systems are named as nanotechnology (1). The speech that was given by Richard Feynman at American Physics Community in 1959 is considered as the beginning of nanotechnology. Feynman mentioned about nanotechnology based events in his "There's Plenty of Room at the Bottom" titled speech (2). Nanotechnology is a type of technology that brings new synthesis characteristics to materials by down to their structures as small as one millionth of a millimeter (3). Today, nanotechnology that gives direction to almost every aspect of human life and industry fields all over the world, also expresses operating activities like measuring, guessing, tracing, building in this scale as well as ability to utilize some basic characteristics of its. Nano is a technical measurement unit and it means billionth of any unit. Nanotechnology is an applied science and technology field that unites many fields like molecular chemistry, applied physics, chemical engineering, biotechnology engineering, mechanical engineering, material science, electrical engineering, and industrial engineering. Application purposes of nanotechnology are as follows; production and analysis of nano-scale materials, understanding and researching of nano-sized structures' physical characteristics, development of nano-sensitive tools, development and finding of proper techniques for bonding the macroscopic and the nanoscopic world.

Basic usage and application field of nanotechnology can be categorized as health (in molecular damage identification and repair like tissue and DNA), industrial, scientific research (in surface characterization and modification, moving of microorganisms, DNA modification), pharmaceutical industry (in controlled medicine audit), safer and longer travelling by car, plane and spacecraft with very little energy consumption, cosmetic industry, keeping foods durable as fresh for a long period of time, construction of cell repair robots, manufacturing clothes that not get stained and wet, manufacturing protective clothes that resistant to blow and bullet in military industry, and recycling of waste materials

(4,5).

According to aforementioned, raising awareness about application of nanotechnology and nanobiotechnology in research and techniques at both crime scene and laboratory in the field of forensic genetics is aimed in our study. In line with the information that gathered by literature search, current state of nanoscience application in forensic genetics has investigated; how nanotechnology serves for interdisciplinary fields like forensic genetics and forensic science in the future has tried to reveal.

Nanobiotechnology

Nanobiotechnology includes biotechnology that states usage of biological systems in the production of product and service that based on science and engineering principles by methods which are not affect human and environment health negatively as well as size that equals to one millionth of a millimeters (6,7). Nanobiotechnology term includes two concepts and first one of them is a size definition: nano, it defines the size that equals to one millionth of a millimeter. Second one is biotechnology term, so it defines the technology-based study area that research and put forth applications that biology and biochemistry-based methods and transforming them into products. Combination of these two terms, nanobiotechnology, on the one hand investigates the nanostructures and nanomachines, namely DNA, RNA, lipids, proteins, polysaccharides, their interactions and movements with each other, taking shape during the billions of years of evolution of the living cell. On the other hand, it plans to imitate these structures and interactions by using materials and structures that are more durable, move faster, and reach the planned target at any time (8,9).

Co-development of nanotechnology and biotechnology as well as information accumulation on molecular biology field very quickly nourish these two developing fields and nano biotechnology research area has emerged. Nanobiotechnology's third area of interest is designing systems and mechanisms that enables gathering information at nano level and research biologic systems at nano scale in molecular biology studies, and transforming them into products. Nanobiotechnology studies open a door for completely new production of micro and nano scaled tools and devices (10–13).Thus, getting biological and biomedical devices smaller and production of new devices are expected in the future within biotechnology industry. Some of the main titles of

research/production areas that aimed by nanobiotechnology can be listed as nano-biomolecular devices and analysis methods, nano-scale cell biology and cell-surface interactions. Researches elongates from nano implants to smart drug delivery systems, from nano-bio machines to nanofabrication of DNA chips for bioinformatic and genomic applications, and stem cell-based tissue engineering.

Electo-chemical Sensors Based on Nano-particles and Carbon Nanotubes

Nanoparticles create the today's latest technology (14). Because nanoparticles provide better measurement performance, easy-to-use, and cheaper price possibilities, they are used in bio-measurements. Besides, nanoparticles can be used as routine in bio-measurements thanks to their large production volume, distribution capacity and commercial availability for a broad community.

There is an increasing interest for nanoparticles, carbon nanotubes and other nano materials in last decade (15). Carbon nanotube modified sensors that designed for analyzes of specific molecules like drug, protein and DNA showed that more sensitive analyzes can be performed (16).

It can be seen that disposable electrochemical sensors developed by using carbon nanotubes, enable more sensitive, reliable and optional electrochemical identification of DNA analysis (17,18).In performed studies, determination of DNA-DNA targeted new compounds interaction by electrochemical sensor technology is aimed. Thus, sensors developed by nano materials can be designed in smaller sizes, DNA can be adapted to chip technology, sensors that intended for chip design for lower cost, faster and more sensitive response can be developed.

According to the results of drug-DNA interaction and sequence selected DNA hybridization determination of electrochemical sensors developed by using single wall carbon nanotubes, it can be seen that these developed sensors can eliminate other complicated and time consuming surface modifications, therefore they can provide more sensitive, reliable and optional DNA analyzes (19,20).

Nano-Scale Cell Biology

Observing and measuring of living cells at nanometer level can be possible by using tools and devices developed by advanced physics researches (21,22). Furthermore, new tools and applications that can do measurement at nano and micro-scale are added to them each day. Therefore, sample analyzes that covers many proteins as well as simultaneous examination of cell functionality and drug effects, and monitoring the impact of drug candidates on living cell functions are now considered within feasibility limits. Nanoparticles, tools obtained as a result of nano-production and molecular design information are used in this and similar studies. Beyond this, cell biology can be investigated with these advanced physics product microscopes and measurement devices as well as physical alterations on DNA at manometer resolution can be observed by atomic force microscope as an example.

Two different approach is seen when studies performed with nanoparticles are observed. In one of them, active molecules are placed in nanoparticles and then they are tried to direct to selected targets (23). Apart from that predictive studies on foreign DNA transfer on plant cells by using carbon nanotubes can be found in literature. In the second approach that nanoparticles are used, increasing of visualization intended for diagnosis with the addition of functional molecules to nanoparticles, and increasing of the treatment capacity by utilization of thermal and chemical characteristics of particles are aimed (24–26).

DNA Chip Technology

Chips are small devices used in electronic industry for years (27). Biochip technology is a type of technology that arise from it. DNA, cDNA or oligonucleotide, protein and so on analysis on small support materials, glass microscope slides, silicon chips or nylon membranes can be performed by microchip (microarray) technology.

Microfabrication is a method applied for creating physical objects that have very small size like mm or μm (27–29).Development of micro and nanotechnology with application on biomedical arena, it has quite big potential on development of new diagnosis and treatment systems. With an improvement of this technology, early identification of illness and risk conditions, providing less harm and less recovery time, and providing more acceptable health-care with less cost can be possible.

These systems have important advantages when compared with devices produced with classic production techniques (30,31).Because they have smaller sized structure (micro-size), surface area/volume rate increases. Having an ability for combining sensitivity on the same and adjacent substrate, signal conditions and activation functions, so it provides electronic and electrical components to join on the system. Furthermore, hundreds of them can be produced as easy as a device, and depending on this serial fabrication, they are produced cheaper. In addition, they are geometrically controllable beside providing super functions like sensitivity and analysis.

Integration of nanotechnology with medicine and biology fields bring along the expectations on molecular diagnosis and treatment fields (28,32). Nanotechnology enables DNA analyzes with a very little sample. Besides, earlier and faster diagnosis of illnesses can be provided by development of this technology.

Almost all of the studies covered by genomic researches can be performed by DNA chips(33–35). Genes in DNA and their sequences can be found. Especially in criminology studies, criminal identification can be done very quickly by DNA test. Tracking the mutations which can emerge during illness status, diagnosis of illness, improvement/regression of illness can be tracked by monitoring changes in gene expression profile by time. By following possible toxic impacts of new drug candidates, their eliminations can be done easily. Interaction mechanism of DNA targeted potential drugs can be tracked on chip.

Nanoscience and Forensic Sciences

In forensic science studies, goal is not giving coincidence place (1,36–38). Besides, minimizing the human effect as possible as, moving validity and reliability of laboratory activities to the highest point. Forensic science laboratories perform the requested analyzes proper for validated methods by using correct equipment and material, and make use of developing technologies. Being better and perfect of the services which presented by forensic science laboratories and forensic science community is possible by following the developed technology. An occurred event not affect just suspect, victim or both of their relatives, a whole society who relates with them are affected indirectly. With the help of nanotechnology, more precise and selective new methods are developing for shaping of forensic expert reports and analyzes of evidences, as well as nano-

kits which have high efficiency used in experimental process are produced (39–41).

Nano-forensic sciences is a new and highly developed forensic science field related with development of nano sensors for crime investigations and examination terrorist activities by determination of exploiting gases, biological agents and presence of remains (1,42). In nanotechnological developments within forensic science field, character of particle features is changed by increasing the rate of 'nanoparticles' as nanometer size and widen chemical diversity (43,44). Nanotechnology and developing nanoparticles within forensic sciences are usually used analyzes like development of biological, ballistic and latent fingerprints, prevention of document forgery crimes, illegal drug identification and quantification, alcohol measurement on drunk drivers, analysis of explosive substances used in explosion case especially in terrorist act, nerve gas identification, inorganic pigment identification in hit-and-run car crash cases, determination of death time, DNA analysis, security, privacy, defense field and so on at both crime scene and laboratories. Even in 'illicit drug' called illegal drug identification investigations which have a lot of samples to be analyzed and each gram of it must be analyzed, reliable, accurate and precise results can be reached in a short time by using nanotechnology.

Nanotechnology has a great impact on modern technology like development of DNA profiling intended techniques on biotechnology in last 50 years (33,36,45). In analyzes performed within forensic sciences, information and techniques from basic science and life sciences as well as determination, identification, and evaluation of evidences are aimed. Evidences are then used for recreation of crime scenes, guiding for investigations and bring criminals to justice. Various nanotechnological techniques named as nano-analysis, do applications intended for these objectives and they are used commonly in criminal determination.

Nanotechnological Applications in Forensic Genetics

In forensic genetics studies, typing of individual is affected directly from successful nucleic acid isolation from biological evidences, and performing PCR well (46).For nucleic acid isolation, many extraction included commercial kits following column chromatography or agarose gel electrophoresis are available. However, these techniques are not successful enough to prevent contamination or complicated procedures. It is very important that

purifying nucleic acids, isolated from biological samples, from any contaminant substance. In recent years, genomic DNA isolation and purification process has significantly made progress as well as improvement in methodologies for DNA isolation is required currently. In order to answer this type of questions and overcome the risks, nanotechnology tend to especially DNA isolation researches lately.

Integrated systems has developed to isolate DNA in systems which are using solid phase extraction principle and get attention thanks to easy manipulation of magnetic nanoparticles and cost effectiveness (47–49). Magnetic nanoparticles are becoming increasingly more popular technique in order to isolation of biomolecules like proteins, DNA and RNA from their environment separately. In various studies, it is stated that success of magnetic nanoparticles for obtaining genomic DNA from body fluids like blood, saliva and semen. In performed researches, success of magnetic nanoparticles which are coated with various polymers like agarose, carboxyl and silica was investigated in DNA isolation. DNA absorbing solid phase systems, silica-based particles, glass fibers, anion exchange carriers and modified magnetics beads are determined that they are not causing DNA degradation or quantity loss during purification. Additionally, more progress can be achieved in DNA analysis field by developing nanotechnology-based tools which can be used directly for reading DNA sequence in a molecule. Besides, DNA sequence can be analyzed with using atomic force microscope technique by placing DNA molecules on a carbon nanotube. Because degree of decomposition and destruction in antic remains or postmortem samples is very high in most of the cases, DNA extraction and isolation by current methods are inadequate. Lodha et all. benefit from unique feature of copper nanoparticles in their studies intended to DNA obtaining from antic skeleton remains in 2016 (50).Copper nanoparticles were synthesized at microwave medium by using diethyl glycol (DEG) which bind to DNA with very high affinity, ascorbic acid and Poli Vinyl Pyrolidon (PVP). The innovative direction of this method is copper nanoparticles are not applied for DNA isolation until now. Besides, researchers reported that this developed method can easily be applied for DNA isolation from skeleton remains. In their research intended for increasing of bacterial DNA quality and quantity in 2015, Muntaha et al. found that bacterial DNA is obtained from

gram negative bacteria by extraction methods like boiling, alkaline lysis, salting in the presence of ZnO nanoparticles (51). In the presence of TiO_2 nanoparticles, improvement in obtained DNA quantity from gram negative and gram-positive bacteria by just using salting method was detected. Kwang et al. used longitudinal resonance of Au nanorods in order to transform close infrared energy into thermal energy on a microfluid chip. It is determined that emerging heat cause pathogen fragmentation effectively. In this study, DNA was removed from inside of the cell and transferred to a PCR system. By this research, it was determined that successful usage of one step real time PCR system for pathogen determination without removing or changing reagents.

Capillary electrophoresis basically consist of narrow capillary, two buffer flacons, two electrodes, high voltage power supply, florescence detector (laser excitation source), automatic sampling for loading the samples automatically, and a computer in order to control every step. Capillary electrophoresis system is a tiring technique for researchers because of the capillary length, freezing of polymer in inside as a result of environmental conditions, getting effected by electrical waving, cost, speed, and limitations about sensitivity.

In forensic genetics, development of micro fluid systems as an alternative to capillary electrophoresis, is the most effective nanotechnological developments for changing the current work procedures in DNA analysis laboratories (48,52,53). These devices are more compact than a standard capillary and gel electrophoresis systems. Short analysis time, lowering of contamination risk, providing service directly at the crime scene, being in small size and portable, easy cleaning and caring are mentioned advantages of these devices in various researches.

An innovative concept called lab-on-a-chip (LOC) has developed by the progress in nanotechnology (53–57). LOC technology combines one or more laboratory function on a single chip (sizes varying millimeter from several centimeter) which able to hold very little sample quantity (able to hold very little liquid volume up to pico liter). All laboratory processes can be integrated in LOC in order to perform chemical analysis, so manual intervention is minimized. Related with this, various commercial systems like Gene Chip, Bios Chipset are under development, and these methods enable whole DNA isolation, amplification, separation of amplified products, and

creating DNA profile in place, creating a portable system based on rapid DNA analysis. Taking a biological sample from crime scene, creating this sample's DNA profile and loading it to the database are actualized in a short time like 90 minutes. Thanks to nanotechnology-based sequencing, it is possible that hundred thousands of DNA molecules can be sequenced as parallel and forming DNA profiling rapid and effectively.

Because quantum mechanics are commonly used in nanoparticles, interaction between light and substance become important (58–60). To illustrate, these characteristics lead to beneficial materials like quantum nanocrystals which produce intense florescence and have none of the spectral band expansion because of the diversity of different electronic energy levels in dye molecule. Thus, current problems with the matrices of florescent dyes used in visualization of DNA, can be reduced in a large scale. DNA molecules can be labeled one by one with these materials and more different color features can be produced by simply changing the size of nanocrystal.

Nano-scale production methods are usually contains same lithography techniques used in silica based computer chips (61). These techniques are also used to synthesize short single strand DNA fragments for producing wide sequences consists of more than 250.000 DNA single nucleotide polymorphism probes on a single silicon chip. Additionally, lithographic procedures can be used decreasing the size of chemical instrumentation like capillary electrophoresis and liquid chromatography. By this way, new analytical devices and systems which can be replaced with chemical instrumentation can be developed. These systems can be used to isolate molecules, performing chemical reactions, separating and detect products. The pyrosequencing technique which produced in this way, is a similar application to PCR system.

The inventions brought by nanotechnology is much more comprehensive and robust when compared with other inventions in science history until now. Hundreds of nanotechnological products like self-cleaning paints, dirt free fabrics, flexible but more resistant concretes, coverings as hard as diamond, agents which kill cancer cells without harming body, creams which not lose their effects for days, sensors that even detect single anthrax microbe, microbe-free refrigerators and so on has come into our lives and continues to enter. Nobel Prize winner Horst Stormer described this area as "

Nanotechnology gave all the required tools to us. Thanks to the most developed toy box of nature, we can play with atom and molecules. What we can do seems infinite". The most widespread comment related to nano science can be described as measuring, tracking and producing on atomic and molecular size, and processing new features in these sizes (62).

Nanotechnology has great potential to benefit for society and forensic science (63). The most important advantage of nanotechnology that advanced analysis techniques are taking part of it in forensic sciences field with both developed and to be developed nanoparticles usage is reaching the evidences which can help for solving the cases that routine and standard research methods are insufficient for lighting up the case by necessary investigation and developing analysis techniques. With the great potential of nanotechnology about benefit for society and forensic science, developed/to be developed new and features unknown nanoparticles can also create a risk in terms of environment and health. In addition to this disadvantage, the most important advantage of nanotechnology usage that involves advanced analysis techniques in forensic sciences field is revealing confidential evidences which can help solving the cases that have unknown perpetrator.

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SUDDEN DEATH DUE TO A CYSTIC LESION IN THE CEREBELLUM. A CASE REPORT

Chrysavgi Kousi, Antigony Mitselou

Cysts of the cerebellar hemispheres are well-known and are caused by cerebellar cystic astrocytoma, cerebellar hemangioblastoma, or angiomas of the cerebellum [1-3]. In each of these lesions, the wall of the cyst typically contains a mural nodule where many tumor cells and blood vessels are encountered. The remainder of the cyst wall is covered by gliotic astrocytes overlying white matter. Surgical resection of the tumor usually cures the lesion. If left untreated, the continually expanding cystic lesion can cause obstruction of the IVth ventricule and obstructive hydrocephalus, brain stem compression, and/or secondary brainstem hemorrhages because of increased intracranial pressure [1, 4].

Pilocytic astrocytoma (PA) is a well-circumscribed, well-differentiated, slowly growing tumor, corresponding to WHO grade I [5]. PA occur most commonly in pediatric and young adults, constituting 10% of cerebral and 85% of cerebellar tumors. PA may arise along the entire neuraxis. They affect preferentially the cerebellum but may arise in the optic pathway, hypothalamus, brainstem, and spinal cord [6]. PA histologically exhibits biphasic pattern with a mixture of loose microcyst and more compact regions [7]. PAs are generally associated with excellent prognosis and favorable long-term outcome or spontaneous regression [8]. Dissemination of cerebellar PAs can be associated with obstructive hydrocephalus leading to an unfavorable prognosis [1, 4].

Here, we describe a very rare case of sudden and unexpected death due to clinically undiagnosed cystic cerebellar pilocytic astrocytoma in a young man. We discuss the mechanisms of death and present a short review of the literature.

The present research was conducted according to the Ethical Guidelines for Medical and Health Research Involving Human Subjects. This study was approved by the Ethics Review Committee of University of Ioannina (approval No. 515α/11-11-2003).

A 24-year-old man had been found unconscious in bed at home. He was rushed to the emergency department of the General Hospital of Corfu, but he died at his admittance. Approximately 1 month before his dead, the patient had consulted a doctor complaining of headache, neck pain, dizziness, nausea, and ataxia. The physician attributed the symptoms in expending many hours in his personal computer (PC).

A forensic autopsy was conducted the day after the patient's death. A well-developed, well nourished, 24-years old white male. Externally, no significant injury or trauma was found. Internally, the heart was of normal size and configuration with no evidence of anomaly, while the lungs showed mild to moderate congestion and pulmonary edema. With exception of the brain, the remaining organs were apparently healthy, with no evidence of macroscopic pathology. The brain weighed 1570 g and showed diffusely cerebral edema and swelling. The coronal sections of the cerebrum showed moderate enlarged lateral ventricules. The axial sections of the cerebellum and brainstem showed a 6.2x6.0x3.4 cm cystic cavity in the right cerebellar hemisphere and a midline shift from right to left (Figure 1). The medulla oblongata was distorted because of transforaminal herniation. The cyst was filled with clear yellow fluid. The inner wall of the cyst was smooth, and a round tumor-like lesion was found (1.5x1x1cm) in the anterolateral wall of the cyst (Figure 2). Focal hemorrhages and necrosis were found in cerebellum and brainstem parenchyma.

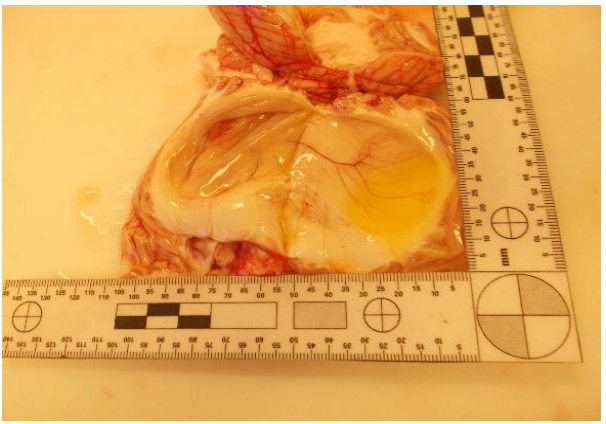

Figure 1: Macroscopic view of the cerebellar cyst. There is an approximately 6.2x6.0x3.4 cm round-shaped cystic cavity in the right cerebellar hemisphere.

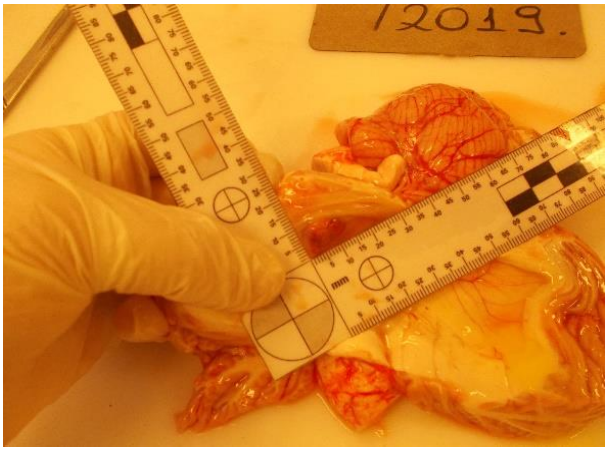

Figure 2: Macroscopic view of the nodular lesion. There is an approximately 1.5x1x1 cm lesion in the in the anterolateral wall of the cyst.

Microscopically, the tumor lesion consisted of a mixture of loose (mixoid) microcystic and some more compact regions. The loosed areas contain astroglial cells and the more compact areas are composed of bipolar piloid cells, with a highly monomorphic, and scanty Rosenthal fibers (Figure 3). Numerous small cysts of different shape and size, filled with eosinophilic myxoid material are observed (Figure 4). The blood vessels are abundant and their walls present fibrosis or hyalinization. In some areas the tumor cells infiltrate the white matter of the cerebellum. The tumor cells are glial fibrillary acidic protein (GFAP) immunopositive (Figure 5). The Ki67 proliferative index is <1%.

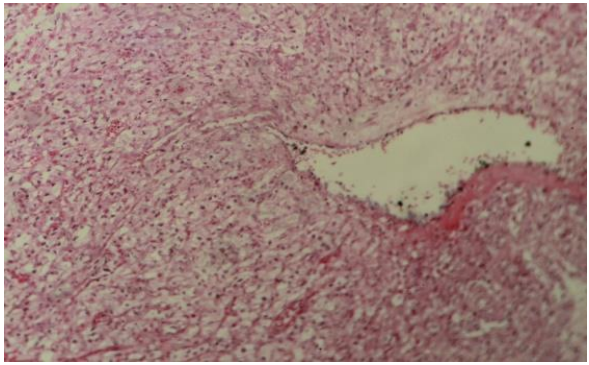

Figure 3: Biphasic architecture with loose spongy component and little compact, fibrillary elements and scattered Rosenthal fibers (H-E x100)

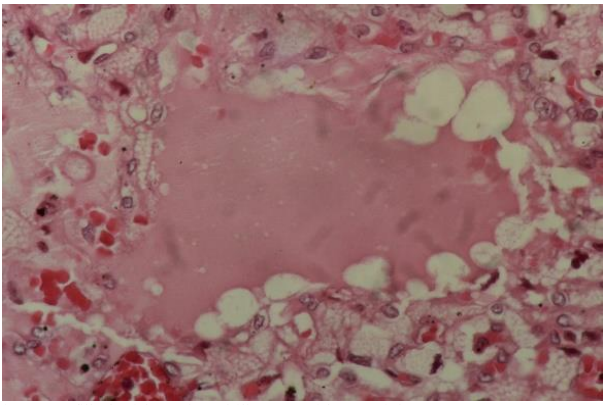

Figure 4: Microcyst filled with eosinophilic, myxoid material (H-E x400)

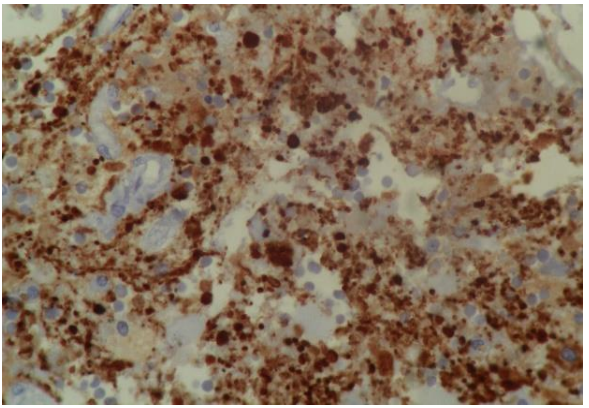

Figure 5: GFAP immunostaining (x200)

Toxicological findings on drugs screening using Triage (Biosite Diagnostic Inc, San Diego, CA, USA) and gas chromatography/mass spectrometry were negative. Blood alcohol was not detected.

In cases of sudden, unexpected death of an apparently healthy individual, the role of the Forensic Pathologist comes to the forefront to arrive at a cause and manner of death. Unexpected and unexplained case of sudden death in young individuals has the greatest impact on the surviving relatives and family members. [9]. Sudden unexpected deaths from intracranial neoplasms are rare. Reports have shown frequencies from 0.02% to 2.2% in medicolegal

autopsies [10].

Cysts of the nervous central system (CNS) can be classified into two categories. The first one includes cysts derived from the CNS and the second cysts derived from non-CNS tissues such as endoderm or ectoderm [1, 4]. Some of the cysts derived from CNS tissue comprise the cysts resulting from damage to CNS tissue, arachnoid cysts, ependymal cysts, neuroectodermal cysts, cysts of the cerebellar hemispheres [1, 4]. Cyst with a mural nodule tumor comprises pilocytic astrocytoma, hemangioblastoma, pleomorphic xanthoastrocytoma, ganglioglioma, desmoplastic infantile ganglioglioma, metastases, and rare cases as tanycytic ependymoma, vascular lesions and neurocysticercosis [4]. Cysts in the cerebellar hemispheres are well known and are cause by cerebellar cystic astrocytoma, cerebellar hemangioblastoma, or sometimes, focal angiomas of the cerebellum [1-4].

Pilocytic astrocytoma (PA) is classified as grade I astrocytic tumor according to the World Health Organization (WHO), accounting approximately 5-6% of all gliomas. PA is most common during the first two decades of life, however, may occur at any age. PA is the most common glioma in children and young adults without sex predilection. PA arise through the neuraxis. Preferred sites include the optic nerve, optic chiasm, hypothalamus, thalamus, basal ganglia, cerebral hemispheres, cerebellum, and brainstem [5]. PA is a slow-growing tumor and common presenting symptoms for cerebellar tumors include ataxia, cranial nerve defects and signs of increased intracranial pressure (headache, nausea, and vomiting), as in our case.

Macroscopically, PAs are generally relatively soft in texture and gray on sections of fixed specimens. They appear to be well-defined. Cysts are common both within the tumor tissue as well as around the tumor, the latter resulting in a cyst with a tumor nodule, as in our case. Calcium deposits and hemosiderin may be present, the latter secondary to small bleeds into tumor tissue [5].

Microscopically, PA is a tumor of low to moderate cellularity with compact, densely fibrillated areas rich in Rosenthal fibers, consisting of cells with long bipolar processes and elongated cytologically bland nuclei, as well as, loosely textured areas, composed of multipolar cells, with bland-to-oval nuclei, and multiple, relatively short cytoplasmic extensions. These areas have varying degrees of mucoid

background material with microcyst development being common, as are also eosinophilic granular bodies or hyaline droplets. In some cases, rarely the tumors are composed predominantly or even entirely of one growth pattern [5]. They display either microcyst or solid appearance with or without eosinophilic granular bodies or Rosenthal fibers. Cells with pleomorphic nuclei, often multinucleated, may also occur and generally are found in the loose microcystic regions. Microvascular proliferation, resulting in relatively thick-walled, hyalinized and/or glomeruloid vessels is often seen, and infarct-like necrosis can occur in some cases [5, 11]. Immunohistochemically the neoplastic cells of PAs show strong GFAP immunoreactivity, S-100 protein and OLIG2 positivity; p53 protein staining is weak to absent and the Ki-67 proliferation index is low in most cases [5].

Recent research has resulted in considerable advances in molecular diagnosis. It was found that constitutive activation of RAS/RAF/MAPK signaling appears to be an important oncogenic process in sporadic PA. The most frequent abnormality found in PAs is the duplication of 7q34, encompassing the BRAF gene. This is a tandem duplication resulting in a transforming fusion gene between KIAA1549 and BRAF. The N-terminus of KIAA1549 protein replaces the N-terminal regulatory region of BRAF, retaining the BRAF kinase domain, which is consequently uncontrolled and constitutively activates the MAPK pathway. This abnormality is most frequent in cerebellar tumors and somewhat less common at other sites [5, 12-14].

Pilocytic astrocytomas are typically well-circumscribed, as in our case, slowly growing WHO grade I tumors that are associated with excellent prognosis, favorable long-term outcome, or spontaneous regression. Surgical remove is the treatment of election [8, 15].

When clinical symptoms are present, the diagnosis of cystic pilocytic astrocytoma is based in computed tomography and magnetic resonance imaging [16]. PAs are detected in CT scans as well-demarcated tumors with both solid and cystic component. MRI has been used with better accuracy than CT. In T1-weighted sequences, the solid portion of the cyst with a mural nodule tumor is iso/hypointense to gray matter while the cyst content is iso- to slightly hyperintense to cerebrospinal fluid. In T2 and fluid attenuated inversion recovery (FLAIR) sequences, the cyst content is not suppressed, and the tumor may show high signal intensity [16].

In some cases, patients with pilocytic astrocytomas may present with a spontaneous intratumoral hemorrhage. Previous studies have demonstrated that spontaneous bleeding in PAs may be attributed to the pathological changes in vascular wall, tumor growth, intratumoral vascular invasion and homeostatic abnormalities [17].

A particular clinicopathological presentation of PAs might be associated with ovarian epidermoid cyst [18]. Reports on astrocytic tumors arising in mature cystic teratomas are extremely rare, the neuroectodermal component undergoing malignant change being a highly exceptional event. Astrocytic and ependymal tissues, nerve ganglia of sympathetic type, and nerve bundles accompanied by Schwann cells may be seen within mature teratomas as well as cavities lined by choroid plexus, containing cerebrospinal fluid [19].

Differential diagnosis includes pleomorphic xanthoastrocytoma, diffuse astrocytoma and glioblastoma or even piloid reactive gliosis typical for long-standing, non-neoplastic pathological processes. Distinction of all these lesions has important therapeutic and prognostic implications [5, 11].

The autopsy revealed a large cystic formation in the right hemisphere of cerebellum, moderate hydrocephalus, and transforaminal herniation. We concluded that the cause of death was attributable to the brain compression which was caused by obstructive hydrocephalus secondary to the mass effect due to pilocytic astrocytoma.

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CASE OF FATAL MESENTERIC THROMBOSIS DUE TO MISSED DIAGNOSIS. FORENSIC DATA REVIEW

Rossen Hadjiev, Margarita Tankova, Georgi Gergov

Mesenteric venous thrombosis (MVT) is a rare condition of acute abdomen. It occurs when a blood clot is formed in major vein(s) that drain blood from the intestines. It can lead to life-threatening complications without prompt treatment. The diagnosis is often challenging because of the variety of symptoms associated with acute abdomen. The causes of MVT include abdomen trauma, genetic disorders with increased risk of blood clotting, appendicitis, inflammatory bowel diseases, such as diverticulitis, ulcerative colitis, and Crohn's disease, pancreatitis, liver disease and cirrhosis, cancers of the digestive system, etc.

MVT decreases the blood supply to the tissues (ischemia), causes intestinal infarction and peritonitis. It is life-threatening condition and requires emergency medical attention.

We present a case from our forensic practice of an 80-year-old man died from a mesenteric thrombosis since the diagnosis was missed from the medical crew in the hospital.

An 80-year-old man fell inside a bus as the driver underwent a sudden braking. He was hospitalized and diagnosed with a pertrochanteric fracture. He was in sufficient general condition, in conscious, afebrile. Examination of the abdomen determined no pathological symptoms, negative Blumberg's sign, and physiological peristalsis. His left leg was shortened, and externally rotated. Abdominal ultrasound examination did not establish any pathological changes of the parenchymal organs. A successful blood repositioning and metal osteosynthesis on the leg was performed, and four days later the patient was moved to a rehabilitation center for palliative cares. Two weeks after the accident acute abdomen symptoms occurred: poorly localized abdominal pain, diarrhea, bloated abdomen, difficult palpation, nausea. The patient was moved into a hospital for active treatment. A diagnostic testing was performed. The laboratory results indicated an ongoing generalized infection and increased coagulation markers. Abdominal ultrasound examination and computed tomography were performed with non-

specific imaging data for acute abdomen. Although applied conservative treatment, the patient died in two days. A prosecutor ordered an autopsy because of the possible connection between the bus accident and the death.

On the next day, an autopsy was performed. The external examination determined a cold corpse and presence of rigor mortis. There were fixed purplish-red post-mortem patches on the posterior uncompressed surfaces of the body. The eyelids were tightly closed, and the eyeballs were properly positioned into the orbits. No visible traumatic injuries of the neck area were established. A decubitus ulcer was established on the waist and an operative suture in the hip joint area. The internal examination determined swollen and livid in color caecum, colon ascendens and colon transversum, as well as a significant part of the small intestine. In some areas along the serosa, single fibrinous deposits were found. Opening of the small intestine revealed blood-like content in the lumen. Opening of the abdominal aorta and iliac vessels determined pathological changes in the intima with multiple ulcerated atherosclerotic plaques.

Tissue samples were taken for additional evaluation. The histological examination confirmed the macroscopic findings. Hemorrhagic infarction of the whole intestine, transmural inflammatory infiltrates, areas of ischemic necrosis of the intestine wall and diffuse fibrinous-purulent peritonitis were established.

The autopsy and the additional histopathological examination revealed that the cause of death was multiple organ failure as a result of mesenteric thrombosis, intestinal infarction and diffuse peritonitis.

In addition to the development of mesenteric thrombosis, an object of interest is the possible connection of the received blunt force trauma to the disease development, the delayed diagnosis of the process and respectively its properly treatment. The neglected data from the examination and presence of clinical symptoms as well as not-performed laparoscopic exploration are an omission of the physician.

With an autopsy performed the forensic medical specialists made the conclusion that there is no causation between the accident and the cause of death. The patient's medical history included myocardial infarction, atherosclerosis, hypertension, dyslipidemia, and chronical kidney disease, which were additionally aggravating factors to the lethal outcome.

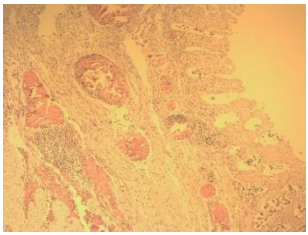
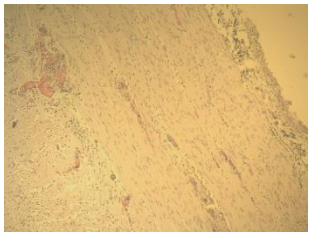


Image 1



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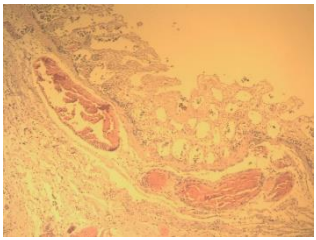

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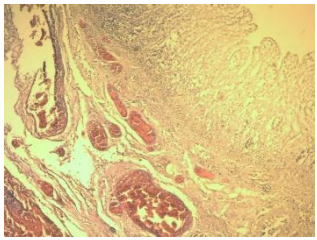

Image 4

Images 1,2,3,4: Hemorrhagic infarction in the intestine wall, areas of ischemic necrosis, hemorrhagic dissection of lamina propria; fibrinous-purulent peritonitis

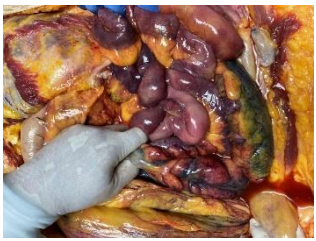

Image 5

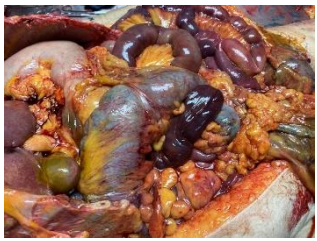

Image 6

Images 5,6: Macroscopic findings during the autopsy. The small and large intestines are inflated, livid in color with hemorrhages.

Each clinical case should be managed particularly and in considering to the patient's specific heath conditions. This ensures reliability for the absence of medical malpractice and an unfavorable outcome for the patients. It is a mandatory condition for implementing a good clinical practice.

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A BULLET INJURY: RICOCHETED OR NOT?

Saime Gizem Tezgel, Şeyma Tuğçe Ünaldı, Nadir Arıcan, İ. Özgür Can, Çağlar Özdemir

During a shooting incident in an indoor or an outdoor urban environment, different types of target surfaces can be hit (1). When hitting a surface, the bullet might penetrate, perforate or ricochet (2). For both solid surfaces and water there is a critical angle of impact (incidence) below which a bullet striking the surface will ricochet rather than penetrate. The critical angle is determined by the nature of the surface, the construction of the bullet, the velocity of the bullet. If the angle of incidence is greater than the critical angle, the bullet either penetrates the surface or breaks up (3).

This case was presented to emphasize the importance of crime scene investigation and reconstruction on the claim of bullet ricochet. The main point of the case became to clarify that it was a ricochet or not.

A 32 years old woman was brought to the emergency unit due to a gunshot injury; a bullet entry was observed under the left zygoma. (In emergency service epicrisis, fire gun entry wound is not descripted in detail). CT scan showed fragmented displaced fractures in the mandible condyle, left temporal, occipital and parietal bones, millimeter-sized multiple metallic foreign bodies in the cranium and a bullet under the scalp in the parietal region. Laceration defined as exit wound in the left parietaoccipital and a bone defect adjacent to this were detected. A deformed bullet, obtained at the surgery sent to criminal laboratory. The case died at the 28th day of her treatment at the intensive care unit and an autopsy was performed. In the autopsy report; 168 cm tall, 45 kg and 30 years old female had an ecchymotic scar of 3x2 cm in the middle of the left cheek, an operation incision sutured in the parietal region of the scalp and possibly a gunshot wound in this region. The autopsy revealed that the death occurred as a result of brain tissue damage and medical complications due to gunshot injury.

The suspect claimed that the gun was shot spontaneously, when he was putting the gun down on the floor while they were sitting face to face with the victim at the corridor.

In the crime scene investigation report; it is stated that there are fresh blood stains (splash-shaped) on both walls at a height of approximately 40-55 cm from the floor, and there is a 9 mm shell on the floor. Beside many blood stains, crime scene investigators reported a suspicious floor defect that may be associated with a ricochet. It has been determined that is approximately 50 cm from the bedroom entrance door.

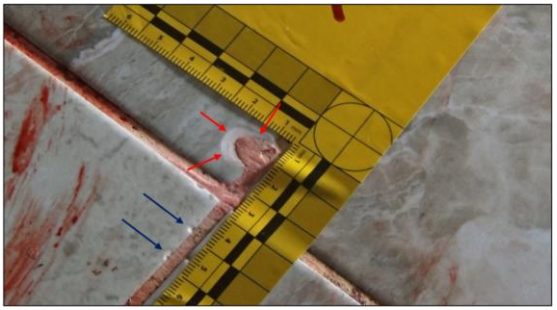

Image 1: Crime scene investigator defined the ceramic damage as a bullet ricochet trace as seen in the photography (showed red lines). Although there is a defect on the edge of the ceramic floor that can be associated with ricochet trace; there are also smaller damages on the side of another ceramic surface (showed blue lines).

This finding indicates that the damage in question also existed during the stage of ceramic tile filling prior to the incident. Since it is not our area of expertise, we cannot comment with certainty on this issue.

Expert witnesses were not allowed to visit the crime scene again, so we reconstructed the crime scene virtually according to suspect's statements. For this process, the "3D Studio Max" software was used, and the 3D images obtained were converted into 2D photographs with the "Vray rendering engine".

Image 2: Considering the standard dimensions of the door and ceramic tile (ceramic tile size is 60x60 cm), the width of the corridor has been drawn as 115 cm (since the tiles on the left have been cut).

The height of the female model used in this animation is 169 cm with her hair, and the height of the male model is 182 cm with his hair. In the position seen in pictures the height of the entrance hole under the left zygoma to the ground was measured as 89 cm.

Image 3: the positions of the suspect and the victim, the alleged position of the gun, the alleged course of the bullet (blue line) as viewed from the bedroom side. If the head is positioned further in the direction of the red arrow, the rebound angle should also increase.

Image 4: During the crime scene investigation, it was understood that the victim had to be in the position shown in the second picture (B) in order for the transfer stain, which was described at a height of 40-55 cm from the ground and observed in first picture (A), to come into contact with the exit wound.

As mentioned by DiMaio, the maximum ricochet angle detected in shots made with 9 mm diameter bullets at steel plates and hard concrete surfaces with an incident angle of 10 to 60 degrees is 5 degrees. Bullets, fired with an incident angle above 30 degrees mostly tend to fragment or penetrate, and also lose 80 % of their energy by an impact with an incident angle over 50 degrees (3).

3D crime scene reconstruction of the case revealed that;

- When the head of the victim was positioned backward against the wall in order to achieve the lowest angle, the ricochet angle may be 63 degrees minimally.
- It is not possible to ricochet off with this angle and penetrate mandibular, temporal and occipital bones with its retained energy.

As a conclusion, considering all evidences together, it was thought that the incident most probably did not occur as a result of the bullet ricocheting off. We understood the importance of interpreting the projectile ricochet cases based on the scientific literature, together with the findings in the crime scene investigation and reconstruction thanks to this case.

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THE HIDDEN POISONING: PHOSPHINE. A FORENSIC CASE REPORT

Silviya Stoykova, Ivo Ivanov, Rositsa Kostandieva, Elka Ivanova, Vasil Atanasov

The cases of phosphine poisoning in Bulgaria are extremely rare and accidental. Most of them are incidents when the restricted (only for professional use) commercial aluminum phosphide products are improperly stored and misused in household.

Phosphine (PH_3) is a colorless, highly flammable gas, with unpleasant odor of garlic or decaying fish, used as an agricultural fumigant. It is usually generated *in situ* by the action of moisture on phosphides (1, 2). The most popular and commercially available phosphide is the aluminum phosphide (AlP). AlP is sold as tablets since 1940s (3). It is popular as fumigant due to its cheapness, efficiency and is widely used as a grain preservative worldwide (4, 5). AlP reacts with moisture (air), water, and hydrochloric acid in the stomach (if ingested) and produces phosphine gas. In human, two routes of exposure to phosphine are common: ingestion of AlP or zinc phosphide and inhalation of phosphine gas. The mortality in cases of phosphine poisoning is very high (60 – 90%) and usually occurs within 24 h. The most common cause of death is cardiac dysrhythmia as well as shock, myocarditis or multiorgan failure (6, 7).

Phosphine is easily distributed to all body tissues. The major metabolites of phosphine are phosphite and hypophosphite (2, 8). The mechanism of phosphine toxicity is associated with respiratory inhibition. It acts as non-competitive inhibitor of cytochrome oxidase in mitochondria, inhibiting oxidative phosphorylation and generating peroxide radicals as a consequent toxic effect (9-11). The oxidative respiration is inhibited by up to 70% and results in a significant reduction in the mitochondrial membrane potential. Another toxic effect is associated with glutathione depletion and catalase inhibition, leading to cellular wall damage (12-15).

Acute human poisoning after phosphine inhalation has a quick onset in a few minutes after exposure, although in some cases has a long latency of up to 48 h. The first symptoms include dizziness, headache, fatigue, nausea, vomiting, abdominal pain, diarrhea. Severe poisoning results in convulsions, pulmonary edema, shock,

coma, significant myocardial and liver damage, kidney failure, etc. (16).

Postmortem findings, usually reported, are pulmonary oedema with hemorrhages in the interlobular areas and the margins of the lung. Lungs, trachea, myocardium, gastric mucosa, kidneys, liver, spleen and brain are congested (8). In histopathological studies different degree of congestion, edema and leukocytic infiltration are observed, suggesting cellular hypoxia in AlP poisoning. The most significant effects are produced in lungs, kidneys and adrenals (17).

The confirmation of phosphine poisoning is usually based on phosphine presence in the biological samples and its metabolites detection. Various analytical strategies could be applied as the simplest one is based on silver nitrate test (18). Another chemical assay is based on the color reaction in presence of different dyes (dimethyl yellow, cresol red) and mercuric chloride (19). However, the most significant method is the gas chromatography (GC) confirmation. There are a numerous analytical applications using GC coupled with different detectors as flame photometric detector (FPD), nitrogen–phosphorus detector (NPD), and mass spectrometry (MS) and using different analytical columns (DB-17, DB-5, Plot Q) (8). A powerful method for detection of phosphine and for confirmation of phosphine poisoning is the conversion of phosphine metabolites by reductive reaction of zinc/sulfuric acid in which the phosphite and hypophosphite generate phosphine gas, measured by GC-NPD (6).

In the present manuscript, we described a case report of fatal phosphine (released from AlP tablets) poisoning, in which the case is solved using phosphine detection in *postmortem* samples, and also the mechanism of poisoning (chain-of-custody of the case) was analyzed to confirm the case scenario.

A 9-year-old boy and his sister (11 years old) went to bed (sharing a bed; in a ground floor room). The family lives in poverty and the children were cared for by their grandparents. A few hours later the boy and his sister display unspecific symptoms (nausea and vomiting). The boy's sister was moved to another room in the house due to the boy's deteriorating condition. The boy was examined and medicated twice from the ambulance service with metoclopramide (at home, in the same room, on the same bed). However, 24 hours later the boy died (at home). Then, his sister was admitted to the intensive care unit (ICU) in the nearest University Hospital. Initially,

there was no data about any pesticide usage at home. All members of the family keep silence about a possible poisoning.

The clinical investigation of the girl showed that all diagnostic tests were found to be negative, but the non-specific heart failure persisted. Then suspicion of poisoning arose. A few days later, the children's grandfather confessed that he had put several tablets of AlP in a rodent-hole under the child's bed, which had been closed with mud. The original bottle of Quickphos ® was found in a septic tank near the house.

The *postmortem* samples were submitted for evidence-based toxicological analysis to prove the phosphine poisoning and to confirm the case scenario of phosphine poisoning.

Chemicals and Reagents

All reagents were of analytical grade and all solvents used - of chromatographic grade. zinc powder (Zn), sulfuric acid - 99.999% (H_2SO_4) and sodium hypophosphite ($NaH_2PO_2.H_2O$) were supplied from Sigma-Aldrich (Germany).

In all experiments deionized water (dH_2O; 18.2 MΩ•cm) was used.

Sample preparation of biological postmortem specimens (2, 6)

40 mg of Zn were placed uniformly in the bottom of a 10-mL headspace vial, and 1 mL of biological sample (blood or urine) or 1 g of tissue (brain or liver), 2 mL dH_2O and 500 μL of H_2SO_4 were added sequentially. Then, the vial was immediately sealed with crimp cap (including silicone septum). The vial was heated at 40 °C for 30 min and, after being held for 10 min at room temperature, 1 mL of vapor-gas phase were injected into GC-NPD.

Instrumental analysis – GC-NPD conditions

Instrumental analyses were carried out on an Agilent 6890N GC system, equipped with a nitrogen-phosphorus detectors (NPD) and fitted with HP-5 capillary column (length 30 m × internal diameter 0.32 mm × 0.25 μm film thickness) (Agilent Technologies, USA) (20). The inlet was operated in splitless injection mode at 150 °C. Helium was used as the carrier gas, flowing at 1 mL/min. The oven temperature program was ramped from 45°C to 110°C at 20°C/min with 2 min hold at 45°C and at final temperature. The NPD was configured with black ceramic bead assembly (greater selectivity for phosphorus) and operated using an H_2 flow rate of 3 mL/min, air at 60 mL/min and N_2 (make-up gas) at 10 mL/min. The detector temperature was held at 320 °C. Bead voltage of the detector was set

to 2.865 V. Data analysis was performed using Agilent HPCORE ChemStation (Agilent Technologies, USA). The retention time of PH_3 was 2.8 min.

Quantitative analysis

Phosphine gas was generated by the reduction reaction of NaH_2PO_2 according to the following equation:

$2\ NaH_2PO_2 + 4\ Zn + 5\ H_2SO_4 \rightarrow 4\ ZnSO_4 + Na_2SO_4 + 4\ H_2O + 2\ PH_3\uparrow$ (6).

Hereof, 80 mg of NaH_2PO_2 was weighted and dissolved in 25.0 mL of dH_2O to obtain an aqueous stock solution. The working standard solution (STD) was prepared by 1:100 dilution in water to a final concentration of 32.0 μg/mL hypophosphite, releasing 10.3 μg/mL PH_3, if a complete and stoichiometric conversion were assumed. For analysis, 1 mL of the STD was used in sample preparation instead of biological sample. The PH_3 quantification was performed by measuring the area of the peak in tested samples and comparing it with that in the STD.

The autopsy of the deceased was performed the same day, a few hours later after his death. During the macroscopic observation, no specific damages to the internal organs were observed according to the medical examiner report.

The histological results are presented in Table 1.

Tissue	***Histopathological changes***
Lungs	Blood stasis and scarce alveolar collections of red blood cells in most alveolar spaces, zones of alveolar edema, single emphysematous swollen alveoli
Heart (Myocardium)	Preserved bundle structure, heavily blood-filled vessels, no clear differentiation between transverse striation and focal granular degeneration of cardiac muscle cells
Brain	Brain parenchyma with preserved structure, blood-filled vessels, mild pericellular and perivascular edema
Liver	Preserved fragmentary structure, blood stasis in the central veins and sinusoids, edematous enlarged liver cells with granular to vacuolar degeneration of their cytoplasm
Kidney	Blood stasis, enlarged glomeruli with narrowed Bauman spaces, granular and vacuolar degeneration of the proximal tubules

Table 1: Histopathological changes observed in the tissues of the deceased boy.

The forensic toxicological analysis of the *postmortem* samples using the procedure and method, described in the previous section, shows the results, presented in Table 2. The corresponding values include the total phosphine amount in the samples (phosphine, phosphite and hypophosphite).

Sample	***Phosphine (total amount)***
Blood	1.9 µg/mL
Urine	53.0 µg/mL
Liver	5.1 µg/g
Brain	2.5 µg/g

Table 2: Amount of the phosphine, measured in different postmortem samples

According to the Material Safety Data Sheet (MSDS) of the AlP tablets' manufacturer (Quickphos ®), 1.0 g PH_3 is released per tablet (3.0 g). The chemical analysis of the residual tablets, collected from the crime scene, supposes that the amount of phosphine released was 1.8 g. The dimensions of the room are 3 m × 3 m × 2.5 m, so the concentration of the phosphine in the air inside is calculated on 80 mg/m^3. Immediately Dangerous to Life or Health value (IDLHs; Center for Disease Control, USA) for phosphine is 70 mg/m^3. The child spent inside more than 20 hours.

In the present case, a severe phosphine poisoning in a child is reported. The hidden information from the family leads to wrong diagnosis and underestimation of the clinical status of the child. The boy was treated in an improper way by the ambulance medical service. The fact that another child (the boy's sister) was transferred in another room actually saves her life as the exposition to the phosphine was cancelled.

The common clinical signs of the phosphine poisoning (abdominal pain, fatigue, nausea, vomiting) could be wrongly attributed to frequent enterovirus infection, including food-poisoning. The low rate of phosphine incidents in the country excludes phosphine poisoning from the differential diagnosis of unknown clinical status, even in agricultural area.

Actually, up to the moment in which all laboratory tests of the boy's sister displayed negative results for bacterial or viral infection as a cause of the myocarditis, the hypothesis for poisoning were neglected from the investigation team. When the information about possible poisoning was available and the source of phosphine was

discovered, the necessity of poisoning confirmation was aroused. It is important that acute phosphine poisoning have to be diagnosed in a complex manner, according to the history of exposure, clinical examination, on-site investigation, exclusion of other diseases, and it is rather difficult to diagnose phosphine poisoning in children (21). When the initial information about history of ingestion/inhalation of AlP/phosphine is missing then more specific diagnostic methods need to be applied (18, 22).

In the current case the route of exposure is inhalation. In a relatively small room on the ground floor, the concentration of the phosphine gas is extremely high in the lower level of the room, where the bed of the child is situated. At the same time, phosphine continues to form under the child's bed (from mud-clogged hole with tablets of AlP inside).

The autopsy findings in cases of phosphine inhalation (8, 23) are non-specific and couldn't be definitely attributed to phosphine poisoning. Therefore, the chemical confirmation of the poison (phosphine) or its main metabolites (phosphite, hypophosphite) as biomarkers of exposure is crucial for the forensic solving of the case.

In the most of the cases described, the route of exposure is accidental or intentional (suicidal) oral ingestion (24-27). Here it is of interest that the poisoning is developed in a period of time when inhalation route of exposure is presented (prolonged exposition). To confirm the poisoning, the only way was forensic toxicology testing of the *postmortem* samples. As there were a few days between sampling and analyses we were focused on biomarkers determination. The method of Yan et al. (6) allows to determinate the total amount of phosphine in tissue (free phosphine, hypophosphite and phosphite) using reductive condition of nascent hydrogen combined with headspace-GC-NPD. A benefit of the method is the procedure of validation and quantitation using *in situ* generation of phosphine in the headspace vials via the same procedure from sodium hypophosphite standard solution.

The results obtained in our case correspond to the other published (6, 20) and confirmed acute phosphine poisoning. The interpretation of all already available information together with the calculation about the phosphine concentration in the room (based on the residual amount of the tablets) allows us to conclude that the case has been resolved and the mechanism of child's intoxication has been confirmed.

The case report presented illustrates the clinical underestimation of the common symptoms and the necessity of a complex forensic toxicology resolution of an unclear medical and investigational case. However, the forensic toxicologists have to be involved in the case from the beginning and to have an access to all information available (if only *postmortem* sample were sent for toxicology screening, the specific assay for phosphine is not performed). Exposure to phosphine gas, released from AlP tablets, is dangerous and could be cause of death by itself. The progress of the poisoning is very fast, the symptoms are unspecific, there is no effective therapeutic algorithm to resolve the intoxication even in well-equipped hospitals. The AlP accessibility should be restricted, especially for use in households. In our opinion, official health system should restrict the open sales of this pesticide.

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