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Paper-based microfluidic devices on the crime scene: A simple tool for rapid estimation of post-mortem interval using vitreous humour

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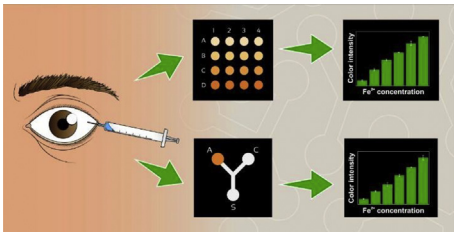
HIGHLIGHTS

- Paper-based devices were proposed to estimate the post-mortem interval using vitreous humor as biological fluid.
- Wax printing technology was used to fabricate a 60-microzone array and a Y-shaped device.
- Digital image analysis provided high specificity, reliability and accuracy.
- The data achieved with paper devices did not differ from those found by ICP-MS at a confidence level of 95%.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper describes for the first time the use of paper-based analytical devices at crime scenes to estimate the post-mortem interval (PMI), based on the colorimetric determination of Fe^{2+} in vitreous humour (VH) samples. Experimental parameters such as the paper substrate, the microzone diameter, the sample volume and the 1,10-phenanthroline (*o*-phen) concentration were optimised in order to ensure the best analytical performance. Grade 1 CHR paper, microzone with diameter of 5 mm, a sample volume of 4 μL and an *o*-phen concentration of 0.05 mol/L were chosen as the optimum experimental conditions. A good linear response was observed for a concentration range of Fe^{2+} between 2 and 10 mg/L and the calculated values for the limit of detection (LOD) and limit of quantification (LOQ) were 0.3 and 0.9 mg/L, respectively. The specificity of the Fe^{2+} colorimetric response was tested in the presence of the main interfering agents and no significant differences were found. After selecting the ideal experimental conditions, four HV samples were investigated on paper-based devices. The concentration levels of Fe^{2+} achieved for samples #1, #2, #3 and #4 were 0.5 ± 0.1, 0.7 ± 0.1, 1.2 ± 0.1 and 15.1 ± 0.1 mg/L, respectively. These values are in good agreement with those calculated by ICP-MS. It important to note that the concentration levels measured using both techniques are proportional to the PMI. The limitation of the proposed analytical device is that it is restricted to a PMI greater than 1 day. The capability of providing an immediate answer about the PMI on the crime scene without any sophisticated

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instrumentation is a great achievement in modern instrumentation for forensic chemistry. The strategy proposed in this study could be helpful in many criminal investigations.

1. Introduction

Several methods have been developed in forensic science for monitoring physical, biological and chemical changes in order to estimate the post-mortem interval (PMI) [1–3]. PMI is defined as the interval elapsed between death and the time of post-mortem examination. This parameter is essential in criminal investigations and it has been estimated based on the analysis of several body fluids such as blood, urine, cerebrospinal fluid, pericardial fluid and vitreous humour (VH) [3–5]. The latter is an aqueous and gelatinous fluid covered by collagen fibrils, composed of 99% water. Compared with other biological fluids, VH offers some attractive advantages including easy affordability, decelerated diffusion and low bacterial degradation [6,7].

In recent years, different techniques such as flame photometry, ICP-MS, LC-MS/MS and use of ion selective electrodes have been explored to estimate the PMI based on the quantification of some species in VH samples [6–13]. The most common species used for this purpose include ammonium [1,8], potassium [1,7–11], sodium [1,6–9], calcium [6–8], magnesium [8], chloride [1,6,7], Fe^{2+} [13], glucose [6,7,12], uric acid [7,12], urea [7], lactate [7,12] and creatinine [6,7]. Recently, Santos Júnior et al. demonstrated for the first time a linear correlation of Fe^{2+} concentration levels in human VH samples with the PMI using the ICP-MS technique [13]. The authors attributed this behaviour to the presence of transferrin and albumin, suggesting a breakdown of the blood-ocular barrier. While the presence of transferrin is related to its ability to capture Fe^{2+} in the eyeball due to decomposition or inflammatory processes, the presence of albumin indicates a passive exudation as a consequence of a conjunctival tissue failure [14,15].

Despite the sensitivity of detection and analytical reliability provided by ICP-MS, the instrumentation required presents some limitations that discourages its use at crime scenes such as high cost and limited portability, as well as a requirement for highly trained personnel. In addition, a laborious sample preparation step is also required. Therefore, the development of straightforward, simple, portable and inexpensive analytical methods is of paramount importance to allow the rapid estimation of PMI, thus enabling its use in criminal investigations. In this scenario, microfluidic paper-based analytical devices (μPADs) appear as promising analytical platforms for chemical analysis in the field, mainly due to their great capability to be coupled with simple colorimetric detectors [16–18]. The advantages of paper microfluidics have contributed to the use of these disposable platforms for recent studies in forensic chemistry. Examples of applications showing the detection of inorganic and military explosives [19–21], explosive residues [22] and procaine in seized cocaine samples [23] have been successfully demonstrated.

In this study, we describe for the first time the use of paper-based devices coupled with colorimetric detection as simple and disposable platforms to estimate the PMI based on the determination of Fe^{2+} concentration levels in human VH samples. The proof-of-concept was investigated using paper microzone array and microfluidic devices fabricated by wax printing technology [24]. The colorimetric reaction was based on the formation of a specific red/orange ferroin complex between Fe^{2+} and 1,10-phenanthroline (*o*-phen). It is important to note that the main drawbacks related to colorimetric detection are attributed to the lack of homogeneous colour development inside the detection zones. In order to enhance the reliability of the colorimetric measurements, we used methacrylic acid to ensure the electrostatic interaction between the negatively charged acid and the positively charged ferroin complex [25].

2. Materials and methods

2.1. Chemicals and materials

Ammonium iron (II) sulphate hexahydrate, hydroxylamine, methacrylic acid, *o*-phen, acetic acid, sodium acetate, sodium nitrite, sodium phosphate, zinc sulphate, copper nitrate, cadmium nitrate, sodium fluoride, nitric acid and hydrogen peroxide were acquired from Sigma Aldrich Co. (Saint Louis, MO, USA). Filter paper (model qualitative, JP 40 and JP 42) was purchased from JProlab (São José dos Pinhais, PR, Brazil). Grade 1 CHR filter paper was obtained from Whatman (Maidstone, Kent, UK). A scanner (model Scanjet G4050) was acquired from Hewlett-Packard (Palo Alto, CA, USA). ICP-MS equipment (model Elan DRC-e) was acquired from PerkinElmer (Norwalk, CT, USA) and closed-vessel microwave equipment (model DGT100 Plus) was acquired from Provecto Analítica (Jundiaí, SP, Brazil). All reagents were analytical grade and were used as received. All solutions were prepared in ultrapure water.

2.2. Fabrication of paper devices

Paper devices were fabricated by wax printing [24]. Briefly, the required geometry was designed on Inkscape™ graphical software and then printed on paper substrates using a wax printer (Xerox ColorQube 8570). Afterwards, the wax printed devices were heated at 180 °C for 120 s on a hot plate to melt the wax and thus create effective hydrophobic barriers. Finally, one side of the device was covered with a clean adhesive tape to prevent the solution leaking and avoid direct contact of the solution with table surface. In this study, two different geometries of paper-based devices were proposed. First, a paper microzone plate (50 mm × 70 mm) was designed containing 60 zones arranged into six columns (1–6) and ten rows (A – J), as depicted in Fig. 1A. In addition, the second geometry was prepared in a Y-shaped format containing three zones (5 mm diameter each) interconnected with a central point by microfluidic channels (1 mm width and 5 mm length) as seen in Fig. 3A.

2.3. Sample collection and preparation for ICP-MS analysis

Sample collection and preparation for ICP-MS analysis were performed according to a previously reported procedure [13]. Briefly, VH samples were collected by Technical Scientific Police agents by puncturing. Then, the material was collected with a 5-mL syringe, packed in grey-top tubes containing 2% (v/v) sodium fluoride and stored at −20 °C to avoid proteolysis. For sample preparation, 200 μL of sample was mixed with 200 μL of HNO_3 and 100 μL of H_2O_2. For sample decomposition, the microwave technique was used with the following program: (1) 60 s at 300 W, (2)

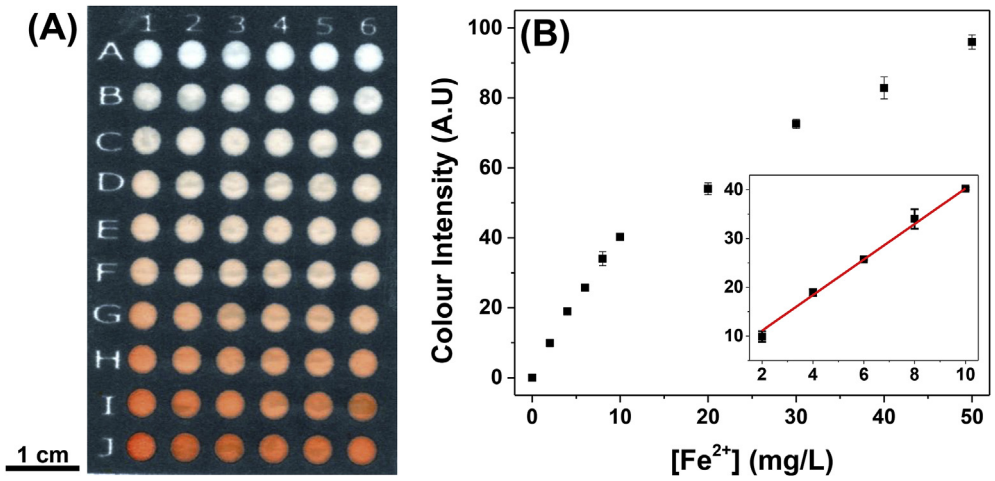

Fig. 1. (A) Optical micrograph showing a colorimetric response for the detection of Fe^{2+} at different concentration levels on paper microzones. Rows A, B, C, D, E, F, G, H, I and J exhibit six colorimetric measurements for Fe^{2+} concentrations fixed at 0, 2, 4, 6, 8, 10, 20, 30, 40 and 50 mg/L, respectively. (B) Analytical curve for Fe^{2+}. The inset graph indicates the linear range of the colorimetric assay. The colorimetric response was recorded after a reaction time of 15 min. Error bars represent the standard deviation value (n = 6).

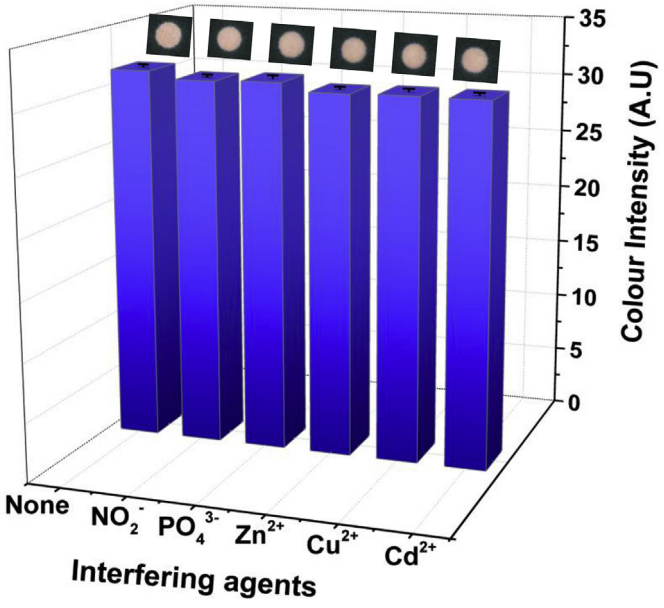

Fig. 2. Colour intensity for colorimetric analysis of Fe^{2+} in the presence of potential interfering agents. The inset images represent the paper microzone in each case after the colorimetric assay. Error bars indicate the standard deviation value (n = 6).

60 s at 500 W, (3) 60 s at 800 W and (4) 60 s at 500 W. After decomposition, VH samples were diluted to 10 mL with deionised water, thus resulting in a 50-fold dilution factor.

2.4. Colorimetric detection procedure

Colorimetric measurements were performed using a benchtop scanner (Hewlett-Packard, model Scanjet G4050) using 600-dpi resolution. The images were captured 15 min after the addition of the standard/sample solution. Then, the digitalised images were analysed in the RGB colour channel using Corel Photo-Paint™ graphical software. The arithmetic mean of the colour intensity within each paper microzone was correlated with the iron concentration and analytical curves were constructed for further quantitative determination in human VH samples.

3. Results and discussion

3.1. Experimental optimisation

In order to understand the behaviour of the proposed paper-based device for Fe^{2+} detection, some experimental parameters including the paper substrate type, the microzone diameter, the sample volume and the *o*-phen concentration were optimised in order to achieve the best analytical response. Initially, four different paper substrates (qualitative, JP 40, JP 42, grade 1 CHR) were evaluated and their analytical responses in terms of sensitivity and

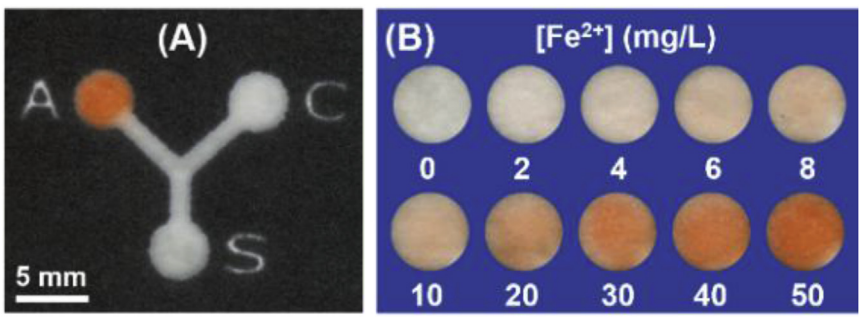

Fig. 3. (A) Optical micrograph showing the microfluidic device used for Fe^{2+} detection under lateral flow and (B) colour scale constructed for rapid visual detection of Fe^{2+} levels in human VH samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

limit of detection (LOD) were compared. This study was quite important as low LOD is required to estimate the PMI by Fe^{2+} quantification [13]. As can be seen in Fig. S1 (available in the Electronic Supplementary Material, ESM), the paper substrate type presented a strong effect on the colorimetric response. Based on the recorded data, the best analytical parameters were provided by grade 1 CHR paper substrate. The achieved LOD and sensitivity were 0.3 mg/L and 6.0 A.U/(mg/L), respectively. Furthermore, this paper also offered the lowest colour gradient. The improved analytical performance promoted by grade 1 CHR paper is associated with its lower porosity, which ensures greater distribution of the sample on the paper [26]. For this reason, this paper type was selected for the subsequent experiments.

The microzone diameter and sample volume were optimised using 10 mg/L Fe^{2+} standard solution. While the microzone diameter ranged from 4 to 7 mm, the sample volume varied from 1 to 6 μL. As can be seen in Fig. S2 (see ESM), the colour intensity was enhanced when the volume increased from 1 to 4 μL. For larger volumes, no significant improvement was observed. Thus, the optimum values for zone diameter and sample volume were 5 mm and 4 μL, respectively. The effect of *o*-phen concentration was also evaluated, as the colour development occurs due to the production of a complex between Fe^{2+} and *o*-phen. This procedure has been described in a previously reported paper, in which the authors obtained the best colour intensity using 8 mg/mL (0.044 mol/L) *o*-phen to form the complex with Fe^{2+} [25]. In the current study, the *o*-phen concentration ranged from 0.05 to 1 mol/L and no noticeable enhancement of colour intensity was observed for the detection of Fe^{2+} at a concentration of 10 mg/L, as shown in Fig. S3 (see ESM). Based on these data, an *o*-phen concentration of 0.05 mol/L was chosen as ideal for subsequent experiments. The optimised conditions were then used to investigate the analytical performance of the proposed device for the determination of Fe^{2+} levels.

3.2. Analytical performance

To evaluate the analytical performance of the proposed paper platform, a calibration curve for Fe^{2+} was obtained. Fig. 1A shows an optical micrograph showing the colour developed in a concentration range from 0 to 50 mg/L. The colorimetric assays were performed by adding 1 μL aliquots of 1.5 mol/L hydroxylamine, followed by 3% (v/v) methacrylic acid and 0.05 mol/L *o*-phen (prepared in ethanol). After each addition, the paper microzones were allowed to dry at room temperature for 5 min.

Fig. 1A displays a colour scale proportional to the Fe^{2+} concentration that is visible to the naked eye. The colour intensity analysis revealed an exponential relationship over a concentration range between 0 and 50 mg/L (Fig. 1B). A good linear relationship (see inset graph) was achieved in a narrower range (2–10 mg/L), which was considered acceptable to demonstrate the proof-of-concept of our hypothesis. The LOD and the limit of quantification (LOQ) values achieved for Fe^{2+} were 0.3 and 0.9 mg/L, respectively. It is important to highlight that the LOD found is lower than the values reported in the literature for colorimetric analysis of Fe^{2+} based on *o*-phen methodology using paper and toner platforms [25,27,28].

3.3. Interfering test

After demonstration of the analytical feasibility of colorimetric Fe^{2+} detection, the specificity of the proposed paper-based devices was examined. The main interfering substances include strong oxidising agents and some metal ions such as nitrite, phosphate, zinc, copper and cadmium [29]. The presence of oxidising agents at high concentrations can interfere with the complexation of Fe^{2+} with *o*-phen. Additionally, the presence of other metal ions at specific concentrations represents a great drawback once they can also form a complex with *o*-phen [29]. Thus, the selectivity of the colorimetric detection of Fe^{2+} was tested in the presence of these potential interfering agents. The colorimetric response was recorded using 10 mg/L Fe^{2+} solutions individually spiked with NO_2^-, PO_4^{3-}, Zn^{2+} (100 mg/L each), Cu^{2+} and Cd^{2+} (5 mg/L each). The colour intensity in each case was compared with the colour signal provided by Fe^{2+} without interfering agents.

As can be seen in Fig. 2, the presence of the interfering species did not induce any noticeable change in the colorimetric response. The pixel intensity values were quite similar and the variation in colour intensity was *ca.* 3.5%. The values obtained were statistically compared by estimating confidence limits for the mean [30], and no statistical differences were observed ($p = 0.05$). Based on the achieved results, it can be inferred that the paper device offers good reliability for use at crime scenes.

3.4. Quantitative analysis of Fe^{2+} in VH samples

The hypothesis related to the use of paper devices to estimate the PMI was demonstrated by colorimetric analysis of Fe^{2+} in four human VH samples. As previously reported, Fe^{2+} is captured by transferrin in the eyeball due to decomposition process [13–15]. Consequently, the longer this stage, the higher the Fe^{2+} concentration levels. For this reason, Fe^{2+} ion was used as a marker to estimate the PMI in VH samples using the proposed devices. Then, the colour intensity was recorded after reaction on paper-based devices and correlated with the iron concentration using the analytical curve. The iron levels obtained for each VH sample were compared to the values calculated by a reference method (ICP-MS). Table 1 displays the Fe^{2+} concentration levels estimated in VH samples using ICP-MS and paper-based devices as well as their relationship with the PMI supplied by Legal Medical Institute from São Paulo Technical-Scientific Police. The concentrations levels of Fe^{2+} achieved by ICP-MS were calculated based on the analytical curve displayed in Fig. S4 (see ESM).

According to the data presented in Table 1, the concentration levels achieved with paper devices are in good agreement with those calculated by ICP-MS. The results obtained with both techniques were statistically compared using a paired *t*-test [30]. As the calculated value ($t = 1.34$) was lower than the critical *t*-test value ($t_c = 3.18$), it can be inferred that the data found by the proposed and reference techniques were not statistically different at a confidence level of 95%. Furthermore, it is important to note that the Fe^{2+} concentrations achieved by both techniques are proportional to the PMI. Thus, the proposed platform can be easily used to estimate the PMI by colorimetric measurements, as the higher the PMI, the greater the Fe^{2+} concentration. While the concentration levels in VH samples #1 and #2 are below the calculated LOQ for paper devices, samples #3 and #4 presented higher values, thus enabling their use for this purpose on the crime scene. An important point to mention is that samples #1 and #2 belong to a control group and samples #3 and #4 were collected from corpses at different stages of decomposition. In this case, the proposed

Table 1
Concentration levels of Fe^{2+} in human VH samples obtained using ICP-MS and paper-based devices (n = 6) and their relationship with the PMI.

VH Sample	ICP-MS (mg/L)	Paper Device (mg/L)	PMI (Days)
#1	0.55 ± 0.02	0.5 ± 0.1	1
#2	0.66 ± 0.09	0.7 ± 0.1	1
#3	1.07 ± 0.05	1.2 ± 0.1	3
#4	14.72 ± 0.12	15.1 ± 0.3	7

methodology is limited to use for samples where the PMI is greater than 1 day. Obviously, the technique could be further improved by changing other experimental conditions not optimised in this study such as pH and reaction time. In addition, it can be noted in the data presented in Table 1 that the relative standard deviation for samples #1 and #2 are considerably higher than those for samples #3 and #4. This is associated with the analytical reliability for measuring Fe^{2+} concentrations lower than LOQ.

To investigate the accuracy of the proposed platform, recovery experiments were performed by spiking a VH sample with three concentration levels of Fe^{2+} (2.5, 5.0 and 7.5 mg/L). The calculated recovery values ranged from 96 to 103%, thus demonstrating suitable accuracy for quantitative analysis.

Besides the paper microzone array, we also investigated the feasibility of a paper-based microfluidic device to determine Fe^{2+} in a lateral flow assay [31]. For this demonstration, a Y-shaped device containing three zones (5 mm diameter each) interconnected at a central point by microfluidic channels (1 mm width and 5 mm length) (see Fig. 3A) was wax printed on paper as described earlier. The zones labelled as S, A and C were used for sample addition, answer and control, respectively. Similar to the paper microzones, the analytical performance was studied (data not shown) resulting in LOD and sensitivity values equal to 0.5 mg/L and 1.9 A.U/(mg/L), respectively. Taking into account the analytical parameters, the LOD values found using the microfluidic device were slightly higher than those reported for paper microzones. The poor analytical response is related to the distribution of the sample through the microfluidic channels towards the detection zones (labelled as A and C). Although the LOD's are higher than those obtained on paper zones, the use of μPADs offers the advantage of the ability to perform multiple assays simultaneously. The incorporation of other target-analytes could represent a helpful tool for the screening of VH samples on the crime scene. The limitations of the proposed μPADs are similar to those mentioned for microzones. Based on a colour scale (Fig. 3B), μPADs were able to distinguish PMI greater than 1 day.

4. Conclusions

In summary, we have successfully demonstrated for the first time the possibility to take and use paper-based microfluidic devices at crime scenes to estimate the PMI using VH samples. The proposed platform is simple, inexpensive, portable and, most importantly, does not require any sophisticated instrumentation to operate and obtains useful information within minutes. Experimental optimisation allowed the selection of paper type and optimal conditions of volume, zone diameter and *o*-phen concentration to achieve the best analytical response. The proposed paper platforms offer high specificity, reliability and accuracy enabling their use in forensic studies through digital image analysis. Alternatively, the platform developed in this study represents a promising and powerful pocket device that could be used in association with a colour scale for police teams around the world. The capability of providing an immediate estimate of PMI at the crime scene could be one of the greatest achievements related to modern instrumentation. This strategy can be helpful to rapidly progress many criminal investigations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.04.040.

References

[1] H.V. Chandrakanth, T. Kanchan, B.M. Balaraj, H.S. Virupaksha, T.N. Chandrashekar, Postmortem vitreous chemistry - an evaluation of sodium, potassium and chloride levels in estimation of time since death (during the first 36 h after death), J. Forensic Leg. Med. 20 (2013) 211–216.
[2] H. Maeda, T. Ishikawa, T. Michiue, Forensic biochemistry for functional investigation of death: concept and practical application, Leg. Med. 13 (2011) 55–67.
[3] C. Palmiere, P. Mangin, Postmortem chemistry update part I, Int. J. Leg. Med. 126 (2012) 187–198.
[4] K.D. Jashnani, S.A. Kale, A.B. Rupani, Vitreous humor: biochemical constituents in estimation of postmortem interval, J. Forensic Sci. 55 (2010) 1523–1527.
[5] R. Swain, A. Kumar, J. Sahoo, R. Lakshmy, S.K. Gupta, D.N. Bhardwaj, R.M. Pandey, Estimation of post-mortem interval: a comparison between cerebrospinal fluid and vitreous humour chemistry, J. Forensic Leg. Med. 36 (2015) 144–148.
[6] B. Madea, F. Musshoff, Postmortem biochemistry, Forensic Sci. Int. 165 (2007) 165–171.
[7] A. Thierauf, F. Musshoff, B. Madea, Post-mortem biochemical investigations of vitreous humor, Forensic Sci. Int. 192 (2009) 78–82.
[8] J.G. Farmer, F. Benomran, A.A. Watson, W.A. Harland, Magnesium, potassium, sodium and calcium in post-mortem vitreous-humor from humans, Forensic Sci. Int. 27 (1985) 1–13.
[9] B. Madea, C. Henssge, W. Honig, A. Gerbracht, References for determining the time of death by potassium in vitreous-humor, Forensic Sci. Int. 40 (1989) 231–243.
[10] B. Madea, A. Rodig, Time of death dependent criteria in vitreous humor - accuracy of estimating the time since death, Forensic Sci. Int. 164 (2006) 87–92.
[11] J.I. Muñoz-Barús, E. Lendoiro, C. Cordeiro, M.S. Rodriguez-Calvo, D.N. Vieira, J.M. Suarez-Penaranda, M. Lopez-Rivadulla, Applications of tandem mass spectrometry (LC-MSMS) in estimating the post-mortem interval using the biochemistry of the vitreous humour, Forensic Sci. Int. 223 (2012) 160–164.
[12] C. Peclet, P. Picotte, F. Jobin, The use of vitreous-humor levels of glucose, lactic-acid and blood-levels of acetone to establish antemortem hyperglycemia in diabetics, Forensic Sci. Int. 65 (1994) 1–6.
[13] J.C. Santos Júnior, P.C. Mollo Filho, R.B.F. Guidugli, M.N. Eberlin, G.S. Pessoa, E.G. da Silva, M.A.Z. Arruda, N.F. Hoehr, Metals and (metallo) proteins identification in vitreous humor focusing on post-mortem biochemistry, Metallomics 6 (2014) 1801–1807.
[14] M.C. McGahan, L.N. Fleisher, A micromethod for the determination of iron and total iron-binding-capacity in intraocular fluids and plasma using electrothermal atomic-absorption spectroscopy, Anal. Biochem. 156 (1986) 397–402.
[15] M. Goralska, J. Ferrell, J. Harned, M. Lall, S. Nagar, L.N. Fleisher, M.C. McGahan, Iron metabolism in the eye: a review, Exp. Eye Res. 88 (2009) 204–215.
[16] D.M. Cate, J.A. Adkins, J. Mettakoonpitak, C.S. Henry, Recent developments in paper-based microfluidic devices, Anal. Chem. 87 (2015) 19–41.
[17] E.W. Nery, L.T. Kubota, Sensing approaches on paper-based devices: a review, Anal. Bioanal. Chem. 405 (2013) 7573–7595.
[18] M. Santhiago, E.W. Nery, G.P. Santos, L.T. Kubota, Microfluidic paper-based devices for bioanalytical applications, Bioanalysis 6 (2014) 89–106.
[19] K.L. Peters, I. Corbin, L.M. Kaufman, K. Zreibe, L. Blanes, B.R. McCord, Simultaneous colorimetric detection of improvised explosive compounds using microfluidic paper-based analytical devices (μPADs), Anal. Methods 7 (2015) 63–70.
[20] M.O. Salles, G.N. Meloni, W.R. de Araujo, T.R.L.C. Paixao, Explosive colorimetric discrimination using a smartphone, paper device and chemometrical approach, Anal. Methods 6 (2014) 2047–2052.
[21] R.V. Taudte, A. Beavis, L. Wilson-Wilde, C. Roux, P. Doble, L. Blanes, A portable explosive detector based on fluorescence quenching of pyrene deposited on coloured wax-printed μPADs, Lab. Chip 13 (2013) 4164–4172.
[22] A. Pesenti, R.V. Taudte, B. McCord, P. Doble, C. Roux, L. Blanes, Coupling paper-based microfluidics and lab on a chip technologies for confirmatory analysis of trinitro aromatic explosives, Anal. Chem. 86 (2014) 4707–4714.
[23] T.G. Silva, W.R. De Araujo, R.A.A. Muñoz, E.M. Richter, M.H.P. Santana, W.K.T. Coltro, T.R.L.C. Paixao, Simple and sensitive paper-based device coupling electrochemical sample pretreatment and colorimetric detection, Anal. Chem. 88 (2016) 5145–5151.
[24] E. Carrilho, A.W. Martinez, G.M. Whitesides, Understanding wax printing: a simple micropatterning process for paper-based microfluidics, Anal. Chem. 81 (2009) 7091–7095.
[25] M.M. Mentele, J. Cunningham, K. Koehler, J. Volckens, C.S. Henry, Microfluidic paper-based analytical device for particulate metals, Anal. Chem. 84 (2012) 4474–4480.
[26] E. Evans, E.F.M. Gabriel, W.K.T. Coltro, C.D. Garcia, Rational selection of substrates to improve color intensity and uniformity on microfluidic paper-based

analytical devices, Analyst 139 (2014) 2127–2132.
[27] P. Rattanarat, W. Dungchai, D. Cate, J. Volckens, O. Chailapakul, C.S. Henry, Multilayer paper-based device for colorimetric and electrochemical quantification of metals, Anal. Chem. 86 (2014) 3555–3562.
[28] F.R. De Souza, G.F. Duarte Junior, P.T. Garcia, W.K.T. Coltro, Avaliação de dispositivos de captura de imagens digitais para detecção colorimétrica em microzonas impressas, Quim. Nova 37 (2014) 1171–1176.
[29] Standard Methods for the Examination of Water and Wastewater, eighteenth ed., 1992. Washington, D. C.
[30] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, sixth ed., Pearson, Harlow, 2010.
[31] A.W. Martinez, S.T. Phillips, E. Carrilho, S.W. Thomas, H. Sindi, G.M. Whitesides, Simple telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-time, off-site diagnosis, Anal. Chem. 80 (2008) 3699–3707.