



# A microfluidic paper-based analytical device ( $\mu$ PAD) with smartphone readout for chlorpyrifos-oxon screening in human serum

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## ABSTRACT

Acute intoxication incidents due to neurotoxic organophosphate (OP) insecticides are occasionally reported, related either to suicidal attempts or occupational exposure due to the misuse of protective equipment. Among them, chlorpyrifos is a compound related to great controversy, which is still authorized and easily accessible in many countries around the world. However, to screen for its exposure markers, instrumental methods are commonly applied, which cannot enable rapid monitoring at an early stage of an intoxication. Therefore, in this study, a microfluidic paper-based analytical device ( $\mu$ PAD) able to rapidly screen for chlorpyrifos-oxon, the toxic chlorpyrifos metabolite, in human serum was developed and fully validated. The  $\mu$ PAD combines wax-printed butyrylcholinesterase (BChE) paper sensors, a lab-on-a-chip (LOC) prototype injector and a smartphone as the analytical detector. In principle, the wax-printed strips with adsorbed BChE are embedded into LOC injectors able to deliver samples and reagents on-demand. A smartphone reader was used to monitor the color development on the strips providing binary qualitative results.  $\mu$ PAD method performance characteristics were thoroughly evaluated in terms of specificity, detection capability (CC $\beta$ ) and ruggedness. The developed analytical platform is rapid (results within 10 min), cost-efficient (0.70 €), potentially applicable at the point-of-need and attained a low CC $\beta$  (10  $\mu$ g L<sup>-1</sup> in human serum). Finally,  $\mu$ PAD characteristics were critically compared to well-established methods, namely an in-house BChE microplate assay and liquid chromatography tandem mass spectrometry.

## 1. Introduction

Organophosphate (OP) insecticides encompass a major pesticide group which have globally found agricultural, industrial, and domestic applications due to their efficiency and low cost. Nevertheless, OPs are neurotoxic compounds inhibiting not only the activity of a vital enzyme for neural signaling, acetylcholinesterase (AChE, EC 3.1.1.7) [1], but also other non-specific cholinesterases such as butyrylcholinesterase (BChE, EC 3.1.1.8), an enzyme used as a biomarker for exposure to OP insecticides [2]. Besides the chronic toxicity risk associated with the potential occurrence of OP residues in foods, there is a significant public health concern related to OP neurotoxicity, especially related to acute intoxication incidents. In addition to their application as warfare agents or in terrorist attacks [3], acute intoxication with OP pesticides is

estimated to cause more than 3 million life-threatening human poisonings each year, and over 250,000 deaths from self-poisoning, which is equal to the one-third of the world's suicide cases [4,5]. Moreover, field workers face OP intoxication threat as well; there were cases [6,7] in which misuse of the protective equipment has been reported resulted in dermal exposure or OP inhalation.

Among OPs, chlorpyrifos is an insecticide related to a great controversy all over the world. An official ban has been applied in the EU due to the European Food Safety Authority (EFSA) concerns predominantly related to neurotoxicity issues [8]. Moreover, according to the latest EFSA report on pesticide residues in food [9], chlorpyrifos was the compound with the most exceedances of its acute reference dose (ARfD) proving that the high concerns related to chlorpyrifos use are pragmatic. In the USA, the Agricultural Health Study reported 588 deaths of

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chlorpyrifos users in a total of 1851 fatal cases [10]. In Asia, chlorpyrifos is considered a major contaminant and can be easily accessed in the market of many countries, e.g. India [11] or Nepal [12]. Furthermore, high mortality rate was reported in chlorpyrifos intoxication incidents (about 4–8%) [13]. Chlorpyrifos neurotoxic potential is expressed only after the metabolic oxidation to chlorpyrifos-oxon by cytochrome P450 enzymes (Fig. 1). Besides oxidative desulfuration forming chlorpyrifos-oxon, diarylation (oxidative ester cleavage) of chlorpyrifos to trichloropyridinol and diethylthiophosphate [14] is included in its metabolic transformation. Chlorpyrifos-oxon detoxification is achieved by A- and B-esterases resulting in trichloropyridinol and diethylthiophosphate, the main detoxification metabolites which are excreted through urine. Consequently, it is necessary to screen for such metabolites to effectively decide on the treatment of intoxicated patients.

Several approaches have been utilized to measure chlorpyrifos metabolites in body tissues [15], with urine being the most common matrix as metabolites are predominantly excreted to this tissue and the urine collection is non-invasive. However, measuring metabolites in urine is associated with certain limitations. Firstly, exposure metabolites excretion lasts up to 48 h [16], meaning that during this period chlorpyrifos metabolites cannot be found in urine. Additionally, in acute intoxication incidents, first symptoms are noticed a few hours after the exposure [16], depending the exposure route (dermal or ingestion). Thus, there is a lack of information at the early intoxication stage. In fact, an early warning of intoxication before symptoms are fully expressed would be helpful to effectively treat such incidents. Moreover, urine metabolites are commonly measured using chromatographic

methods [17,18], which, despite combining sensitivity, robustness and wide linear ranges, are rather costly, time-consuming and need highly qualified laboratory staff and complicated sample preparation. To counter these issues, rapid and cheap diagnostic tests, applicable in other tissues e.g. serum, are urgently needed to act as a complementary early diagnostic tool.

Up to date, several promising alternatives have been proposed for rapid screening of OP intoxication incidents. Ambient mass spectrometry (AMS) methods were developed requiring minimal sample preparation using thermal desorption [19] or paper-spray [20] ion sources. Nevertheless, AMS methods require the use of internal standards for quantification and method cost significantly increases due to the MS-based detection. Alternatively, surface enhanced Raman spectroscopy (SERS) [21] or cholinesterase activity assays [22,23] are considered more affordable choices and have the potential to be applied at the point-of-need. Cholinesterase assays can be useful either using a microplate format providing high throughput or by immobilizing them on a paper surface to exploit paper-based microfluidics advantages, such as portability, disposability, biocompatibility and inexpensiveness [24].

In this study, a microfluidic paper-based analytical device ( $\mu$ PAD) was developed for the rapid screening of chlorpyrifos-oxon in serum samples aiming to provide an early diagnostic tool for intoxication cases. The developed  $\mu$ PAD consisted of BChE cellulose strips functionalized by wax that were utilized as colorimetric sensors. Wax hydrophobic patterns were printed on the cellulose strips creating hydrophilic channels reducing the amount of enzyme that is needed to achieve a colorimetric response. The wax-printed BChE paper sensors were embedded into a

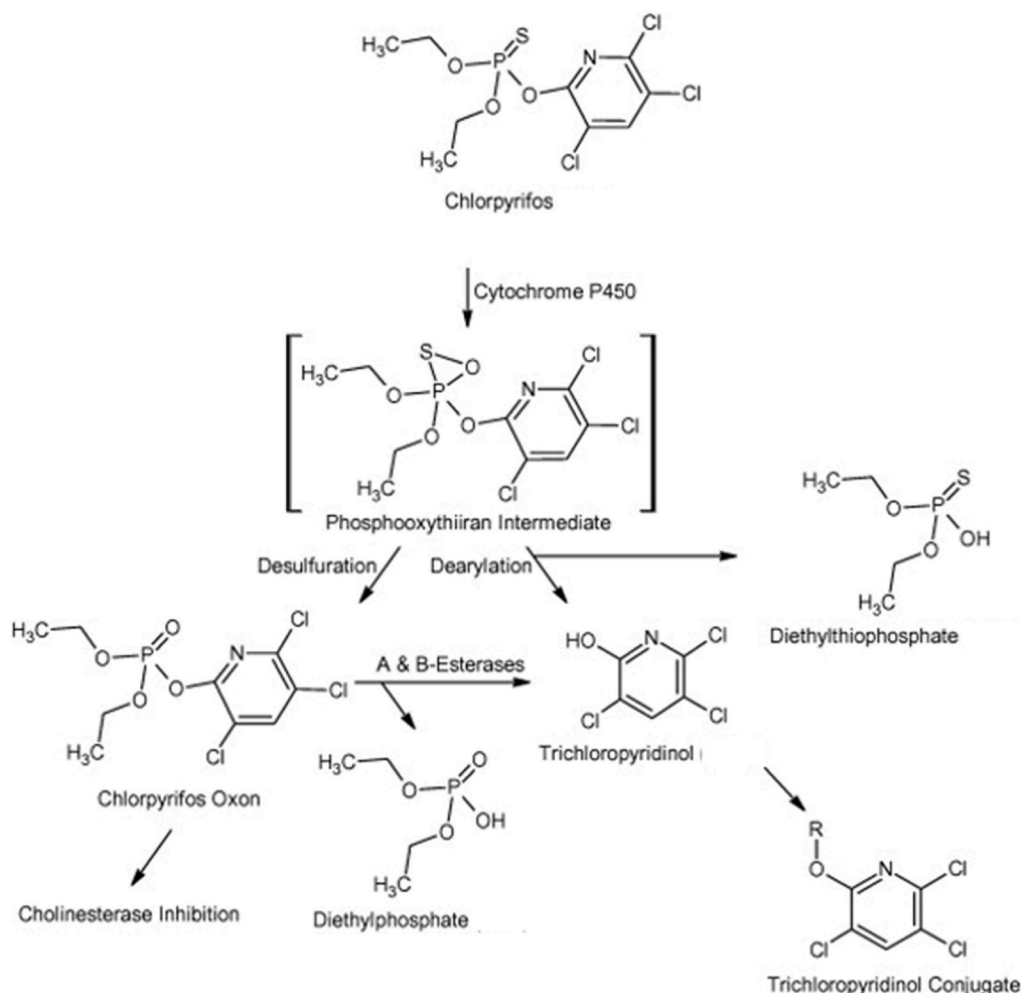


Fig. 1. Metabolic pathway of chlorpyrifos in human organism, reproduced with permission from Ref. [14].

prototype lab-on-a-chip (LOC) injector, which was earlier developed by our group [25], providing integrated sample and reagent handling. This fully portable  $\mu$ PAD was coupled to a universal 3D-printed smartphone reader, able to be attached to various smartphone models, and colorimetric responses were captured by a smartphone camera under fixed illumination conditions creating a comprehensive analytical platform. To provide reliable and repeatable results, the  $\mu$ PAD was fully validated and critically compared towards an in-house BChE microplate assay and a LC-MS/MS method.

## 2. Materials and methods

### 2.1. Apparatus

A stereolithography (SLA) 3D-printer (SL1, Prusa, Czech Republic) was used to fabricate the prototype LOC injectors and the smartphone reader. The printer was controlled by the Prusa Slicer 2.2 software, using Prusa photocurable resin and printing at 100  $\mu$ m layer resolution. The LOC injectors prototypes were designed in our previous study [25]. The smartphone reader consisted of a Huawei P8 lite smartphone coupled to a 3D-printed holder, designed in Autodesk Inventor Fusion for Macbooks. The 3D-printed holder was attached to the smartphone using a plastic clip. In this way, any smartphone having its camera on the top right corner can be used as an analytical detector in this method. The wax-printed paper strips were fabricated using a Xerox ColorQube 8580 wax-printer (Xerox - Norwalk, Connecticut, USA). To select the optimum analytical signal, reflectance measurements were taken using an Ocean Optics 2000 USB optical fiber spectrophotometer (Ocean Optics, Ostfildern, Germany) equipped for spectral reflectance measurements.

### 2.2. Chemicals & reagents

BChE from horse, butyrylthiocholine iodide (BThI, purity >99%), 5,5'-dithio bis-2-nitrobenzoic acid (DTNB, purity >99), phosphate buffer saline (PBS) tablets, bovine serum albumin (BSA), and Whatman® cellulose chromatography paper were purchased by Sigma-Aldrich (Prague, Czech Republic). Deionized water was purified using a Milli-Q system (Millipore; Bedford, MA, USA). 96-microwell plates were supplied by Gama Group (Ceske Budejovice, Czech Republic). BChE stock solution in PBS (2 U  $\mu$ L<sup>-1</sup>) was stored in the fridge at 4 °C. Aldicarb, carbofuran, carbofuran-3-hydroxy, carbaryl, dichlorvos, chlorpyrifos, chlorpyrifos methyl and paraoxon standards were of analytical standard grade and purchased by Sigma Aldrich (Taufkirchen, Germany). Malathion (purity 99%), malaoxon (purity 99%) and parathion (purity 99%) were bought by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Chlorpyrifos-oxon was of analytical standard grade and supplied by Labicom (Olomouc, Czech Republic) and chlorpyrifos methyl-oxon (purity 99%) was purchased by HPC standards GmbH (Cunnersdorf, Germany). Pesticides stock solutions (3 mg mL<sup>-1</sup>) were prepared in acetonitrile and kept in a freezer at -20 °C protected from ambient light. Pesticides working solutions were daily prepared in PBS.

### 2.3. Wax-printing on Whatman chromatography paper

To reduce the amount of enzyme needed for the colorimetric reaction on paper strips, wax-printing technology was applied. Wax hydrophobic patterns were designed using Microsoft PowerPoint 2019. The designed layouts were printed on Whatman paper. Afterwards, the wax-printed papers were heated for 2 min on a hot plate (Sigma Aldrich, Prague, Czech Republic) at 120 °C [26] to achieve full wax penetration through a paper strip.

### 2.4. BChE immobilization

BChE was physically adsorbed on wax-printed paper strips with 1.4 × 0.3 cm dimensions. The wax-printed paper strips were put on a foil

sheet and 10  $\mu$ L of a 0.025 U  $\mu$ L<sup>-1</sup> BChE working solution were pipetted on each strip resulting in a 0.25 U strip<sup>-1</sup>. This enzyme activity is within the normal range [20] of BChE activity in serum when using a 40 times dilution factor (see section 2.9). Prior using them, the strips were dried under room temperature for 2 h. After immobilization, wax-printed BChE strips were kept in the fridge at 4 °C. To define the shelf-life of the wax-printed strips, blank measurements were performed for a two months period and results are presented in the supplementary materials (Fig. S2).

### 2.5. Interferents and cross-reactivity study

BChE is not a selective recognition element as its activity is similarly inhibited by OP and carbamate (CM) pesticides, but inhibitor potency highly varies. Therefore, it was necessary to evaluate BChE specificity towards chlorpyrifos-oxon. To achieve that, an in-house BChE microplate assay was developed following the same principles as other bio-analytical methods for pesticide residue screening [27,28]. The 96-well microplate format was used to provide high-throughput during the optimization stage. It is important to notice that the same BChE stock solution was used to prepare the wax-printed strips during the  $\mu$ PAD validation. Therefore, the obtained data related to BChE specificity using the microplate assay are also applicable to the developed  $\mu$ PAD.

The BChE microplate assay was slightly modified based on a recent paper of our group [29]. In detail, 30  $\mu$ L BChE (0.030 U well<sup>-1</sup>) were incubated with 30  $\mu$ L of an inhibitor for 15 min. Next, 30  $\mu$ L of a 12.5 mM BThI: 1.5 mM DTNB (9:1, v/v) solution in PBS were added and the absorbance was measured at 412 nm after 2 min using an Epoch Biotek Reader (Vermont, USA). The experiments were performed in three independent days, in triplicate and the data were pooled (n = 9 for each calibration point). To obtain the 20% of the maximal and half maximal inhibitory concentration (IC<sub>20</sub> and IC<sub>50</sub>, respectively), a pesticide was prepared in seven different concentration levels (see supplementary material, Table S1). Dose-response with variable slope analysis was performed using GraphPad prism 5.0 software. Measurements were performed in serum solutions to assess BChE specificity towards chlorpyrifos-oxon as BChE is inhibited by various OP and CM pesticides. The cross-reactivity was calculated as follows:

Cross-reactivity (%) = (IC<sub>50</sub>chlorpyrifos-oxon/IC<sub>50</sub>pesticide) × 100, chlorpyrifos-oxon cross-reactivity was set as 100%.

### 2.6. BChE wax-printed paper assay

Immobilized BChE activity was measured using a modified Ellman's assay [25]. Firstly, paper strips were incubated with a sample (see 2.9 section) for 8 min and then a solution of the enzyme substrate and the chromogenic reagent (75 mM BThI: 7.5 mM DTNB, 9:1 (v/v), respectively) was added, and the yellow color development was monitored for 2 min using the 3D-printed smartphone reader. During measurements, the  $\mu$ PAD was put into the smartphone reader to acquire image data under standardized optical conditions.

### 2.7. Smartphone readout

To achieve a standardized image capturing using the developed smartphone-based reader, the free OpenCamera 1.48.1 app was used, which permits the adjustment of exposure, focus and illumination (see supplementary material, Table S2). Pictures of the wax-printed assay were captured at specified time slots, in detail 2 min after adding samples and 2 min after adding enzyme substrate. The whole measuring procedure was time-recorded using a timer.

### 2.8. Image data processing

An Apple laptop (MacBook Air 2017) was used for image data acquisition and processing. ImageJ 1.53a free software was utilized to

extract RGB intensity values following a specified workflow. In detail, images captured using the smartphone reader were uploaded to Google photos storage service and downloaded to the laptop. As mentioned in section 2.7, two images were captured for each measurement. Both images were processed following the described workflow:

- (i) image data were split to R, G and B channels and the B channel was used as the analytical signal; an informed decision based on the reflectance spectrum of the assay is later described (see 3.2.1 section).
- (ii) Two regions of interest (ROI), one for a blank measurement and one for a contaminated sample, were defined with  $30 \times 70$  pixels dimensions.
- (iii) The plot profile, a function displaying the pixels intensity within the defined area, of both ROIs was extracted and the intensity data were saved into .csv files and imported to Microsoft Excel 2019 for statistical processing.
- (iv) Intensities in both photos were averaged and the following formula used to calculate the analytical signal, B channel intensity =  $B_1 - B_2$ , where  $B_1$  is B channel intensity 2 min after adding samples and  $B_2$  is B channel intensity 2 min after adding the enzyme substrate.

## 2.9. Serum samples

Pooled human serum from male donors was purchased by Sigma Aldrich (Prague, Czech Republic). Each donor was tested for and found non-reactive for Hepatitis B, C and HIV antibody by enzyme-linked immunosorbent assay (ELISA). A simple sample preparation protocol was applied based on [20]. Briefly, the pooled serum was 40 times diluted in PBS and put for 15 min into a heated oven ( $80^\circ\text{C}$ ) to eliminate any inherent enzyme activity. Then, diluted serum was left for 30 min in room temperature to condition and was finally used to prepare working solutions.

## 2.10. Validation

The analytical performance of the developed  $\mu\text{PAD}$  was fully evaluated following the Directive 98/79/EC on in vitro diagnostic medical devices and the Decision 2002/657/EC on the performance of analytical methods. In detail, Directive 98/79/EC provides general guidelines for the appropriate quality characteristics that have to be evaluated. Decision 2002/657/EC defines specific validation procedures based on the (i) quantification capability (qualitative or quantitative) and the (ii) type of method (confirmatory or screening method). Considering that the developed  $\mu\text{PAD}$  is a qualitative screening method, specificity, detection capability ( $\text{CC}\beta$ ), and ruggedness needed to be tested. Specificity was evaluated as it is described in section 2.5.  $\text{CC}\beta$  calculation requires high number of tests, therefore 20 blank and 20 contaminated aliquots from the pooled serum were tested. To prepare the contaminated samples, spiking at the concentration level of  $10 \mu\text{g L}^{-1}$  was performed. Working solutions were produced daily prior enzyme testing. Additionally, ruggedness is also evaluated through  $\text{CC}\beta$  as measurements need to be performed in different days. Worthy to notice is that the applied validation plan is suitable also for qualitative screening assays in the food safety [30], a field where the developed analytical platform can be potentially applied.

## 3. Results & discussion

As discussed in the Introduction, chlorpyrifos intoxication incidents are commonly monitored by using urinary exposure metabolites through instrumental analytical methods. Although such approach provides sensitive and accurate results, metabolites secretion in urine takes up to 48 h minimizing the chance for an early treatment of poisoned individuals. Considering that early symptoms can take a few

hours to be expressed and the high cost and complexity of instrumental analysis, an early warning, cheap and rapid diagnostic tool is highly needed. To this end, we developed a  $\mu\text{PAD}$  device with a smartphone readout using BChE as the recognition element. Screening of chlorpyrifos-oxon in serum using BChE is also convenient because BChE is physiologically located in serum tissue. The developed method, which requires just a dilution as the sample preparation, is cheap and provides integrated sample handling due the use of LOC injectors. A smartphone was exploited as the analytical detector to minimize the use of laboratory equipment and increase the point-of-need applicability. Last but not least, the  $\mu\text{PAD}$  was fully validated as a binary qualitative assay providing a yes/no result and critically compared to well-established methods, in detail BChE microplate assay and LC-MS/MS.

### 3.1. $\mu\text{PAD}$ fabrication and function

To develop a practical solution potentially applicable at the point-of-need, a multidisciplinary approach was applied (Fig. 2). The hybrid paper-LOC analytical platform, which was earlier developed by our group [25], was significantly improved by implementing the wax-printing technology and using a different recognition element. Paper strips surface was modified by wax-printing, a fast, inexpensive and environmentally friendly process [31]. Besides these advantages, high throughput during fabrication was achieved as numerous paper devices were printed within a single run. Regarding the recognition element, BChE was physically immobilized on the wax-printed strips. BChE was preferred to AChE as it has been reported a better marker for acute OP intoxication [32,33] as it is more sensitive towards OP pesticides [34,35]. The latter comes in line to our previous findings [29], where the  $\text{IC}_{50}$  values of OPs against AChE were significantly higher compared to the  $\text{IC}_{50}$  values obtained for the same OP compounds against BChE in this study (see section 3.3). However, BChE activity is sometimes considered also a liver function test and for this reason BChE assay results may need to be verified by other assays such as paraoxonase 1 (PON1, EC 3.1.8.1) and gamma glutamyl transferase (GGT, EC 2.3.2.2) in serum matrix [36]. The wax-printed strips were embedded into LOC injector prototypes which were fabricated using a low-cost commercial 3D-printer, without the need of a cleanroom, with an approximate cost of 0.30 € per LOC device. The LOC devices provided sample integration by using four silicone tubings. These tubings acted as finger pumps enabling sample and reagent injection eliminating the need for pipettes. Interestingly, the wax-printed strips also acted as a permeable element to asymmetrically restrict the backward flow, a novel feature of this hybrid  $\mu\text{PAD}$ .

To assemble a  $\mu\text{PAD}$  and proceed with the smartphone optical readout, two wax-printed strips were positioned into the LOC, both sides were sealed by tape and the top side was trimmed to expose the wax-printed strips to the ambient (Fig. S3). With respect to the smartphone-based colorimetric detection, a 3D-printed attachment creating a smartphone reader was necessary to assure standardized lighting conditions. In this way, unified illumination was achieved by constantly using the camera flashlight and adjusting camera settings via OpenCamera app (see supplementary materials). Notably, the analysis time was 10 min, in detail 8 min for sample incubation, a necessary period for enzyme-inhibitor interaction, and 2 min for color development on strips. Within each run, two samples were monitored one blank and one contaminated providing qualitative results related to the presence of chlorpyrifos-oxon. This means that the developed  $\mu\text{PAD}$  could effectively differentiate responses of contaminated serum samples at the level of  $10 \mu\text{g L}^{-1}$  or higher. To monitor the method performance characteristics, the  $\mu\text{PAD}$  device was fully validated at the serum matrix (see section 3.3).



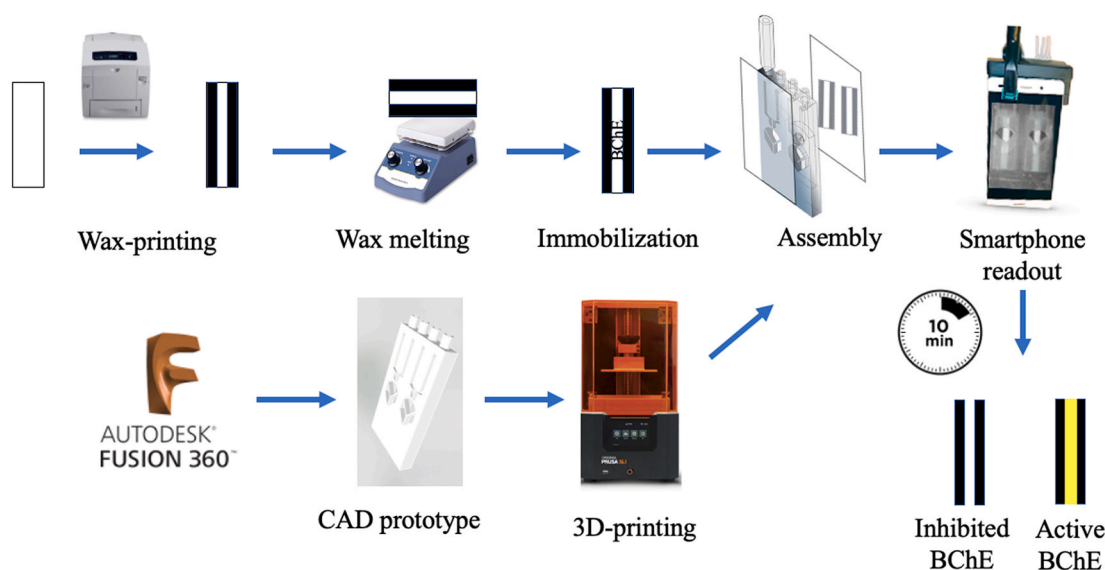


Fig. 2. An overview of the applied workflow to develop the  $\mu$ PAD with a smartphone readout.

### 3.2. $\mu$ PAD optimization

#### 3.2.1. Analytical signal selection

The selection of analytical signal in the smartphone-based optical methods is a controversial topic as numerous color spaces have been used [37], but yet not a clear conclusion has been exported. In contrast to other studies [38,39], which evaluate multiple color spaces and channels without an obvious reasoning, here, a spectral characterization of the wax-printed strips was performed using reflectance spectroscopy to make an informed decision on the analytical signal selection. The reflectance intensity of the  $\mu$ PAD was kinetically investigated by testing a blank sample for 8 min. It was revealed that the response mostly modulates the blue region of the visible spectrum, whereas the red and green band has a flat reflectance (Fig. 3). This means that the analytical information is contained in the blue channel of the RGB color space, and therefore, the blue channel was exploited as the analytical signal during the study. RGB color space is the principal color space as all smartphones capture images using it and further mathematical transformation to other color spaces can actually increase the signal noise. It is important to emphasize that although the described approach is reasonable, easy and without the need of expensive equipment, it is commonly missing from the literature, as this is critically explained in a recent paper from our group [40].

#### 3.2.2. Wax-printing

Initially, it was considered that the wax width on the strip may affect

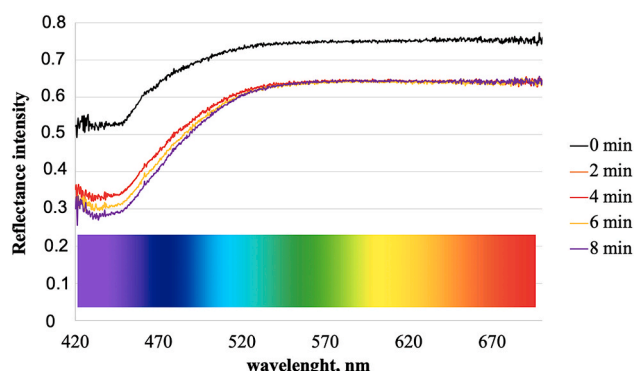


Fig. 3. Kinetic determination of a blank sample reflectance intensity.

the detected color intensity due to different surface magnitude and potential optical contrast due to wax patterns. To investigate this hypothesis, three different types of wax-printed devices were fabricated (Fig. 4a). In this way, hydrophilic channels of 1.5 mm, 2 mm, 3 mm and 5 mm (without any wax) were tested. Importantly, it was not feasible to fabricate functional wax-printed strips with less than 1.5 mm hydrophilic channel due to wax melting during strips heating on a hot plate. A yellow food colorant was used to mimic the color that is developed due to the immobilized enzymatic reaction to avoid any color fluctuations related to the assay. Strips with  $14 \times 3$  mm dimensions (Fig. 4b) provided the highest analytical signal whilst  $14 \times 1.5$  mm devices had similar performance to strips without wax patterns on them (Fig. 4c). Consequently, the  $14 \times 3$  mm wax-printed strips were used for the rest of the study.

#### 3.2.3. BChE specificity towards chlorpyrifos-oxon

Developing useful and sensitive bioanalytical methods impose the utilization of specific recognition elements. Thus, BChE was selected as the bioreceptor as it is irreversibly inhibited by chlorpyrifos-oxon and also it is predominantly found in serum [41]. To investigate BChE specificity towards chlorpyrifos-oxon, a cross-reactivity study was

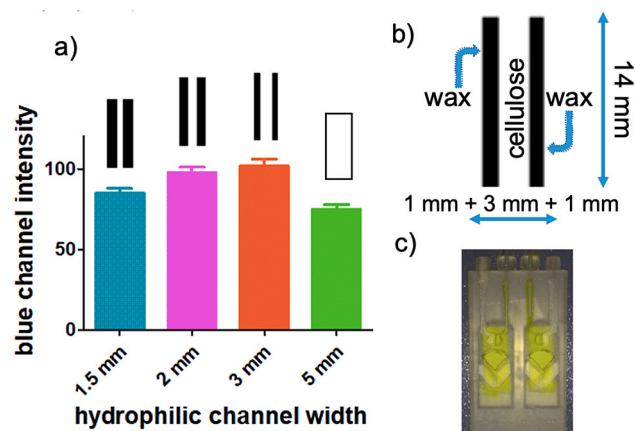


Fig. 4. a) Effect of wax printing on the detected color intensity ( $n = 3$ ); b) Wax-printed paper strip architecture; c) Color testing using strips without any wax patterns. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

performed using an in-house BChE microplate assay. The microplate format provided high throughput during this stage of experiments. Negligible cross-reactivity (<1%) was found towards 11 out of 12 tested pesticides (Table 1) indicating excellent BChE specificity. Chlorpyrifos methyl-oxon was the only of the tested compounds showing a minor cross-reactivity (21%), which cannot be considered as a major drawback since the  $\mu$ PAD method is a qualitative one with a determined cut-off level (see 3.3 section). The strong anticholinesterase effect of chlorpyrifos-oxon and chlorpyrifos methyl-oxon in comparison to other oxons, e.g. paraoxon, was also reported in Ref. [42]. In terms of chlorpyrifos-oxon inhibitory strength, an  $IC_{50}$  value of 0.0035  $\mu$ M was calculated for chlorpyrifos-oxon, which was impressively lower compared to chlorpyrifos, the parent compound (57  $\mu$ M). This comes in accordance to an earlier study, where chlorpyrifos-oxon had three orders of magnitude higher affinity towards the active site of

cholinesterases [43], showing the metabolite significant neurotoxic strength compared to the parent molecule. This is applicable for all the tested thiono analogues (OPs with a P=S moiety) and their respective oxon metabolites, for example parathion (it has a P=S moiety) and paraoxon (it has a P=O moiety). This fact is related to the reduced electronegativity of the P=S moiety in comparison to the P=O which results in a reduced affinity of thiono OPs towards BChE active site.

### 3.3. $\mu$ PAD validation

To provide reliable results and prove the method applicability,  $\mu$ PAD performance characteristics were fully evaluated based on the EU legislation (Directive 98/79/EC & Decision 2002/657/EC, see section 2.10 for further information). Considering that the developed assay is a qualitative screening test, specificity, CC $\beta$  and ruggedness had to be

**Table 1**

BChE specificity towards chlorpyrifos-oxon in terms of cross-reactivity %.

pesticide	structure	$IC_{20}$ , $\mu$ M <sup>1</sup>	$IC_{50}$ , $\mu$ M <sup>1</sup>	Cross-reactivity %
chlorpyrifos-oxon		0.0015 (0.0014–0.0019)	0.0035 (0.0031–0.0039)	100
aldicarb		52 (35–93)	158 (135–192)	0.0022
carbofuran		5.5 (4.5–8.6)	10 (7.3–22.5)	0.035
carbofuran-3-hydroxy		27 (22–42)	62 (40–75)	0.0056
carbaryl		87 (78–98)	142 (112–179)	0.0024
chlorpyrifos		25 (15–43)	57 (41–79)	0.0061
chlorpyrifos methyl		22 (18–27)	75 (64–88)	0.0046
chlorpyrifos methyl-oxon		0.010 (0.0084–0.012)	0.017 (0.015–0.020)	21
dichlorvos		1.3 (1.0–1.6)	2.5 (2.1–3.0)	0.14
malaoxon		8.5 (7.8–9.3)	17 (16–18)	0.020
malathion		84 (75–92)	235 (220–245)	0.0014
paraoxon		1.1 (0.70–1.7)	3.0 (2.1–4.2)	0.11
parathion		126 (110–158)	362 (295–386)	0.0010

\*1: 95% confidence intervals in brackets.

evaluated. With respect to specificity, this was evaluated during method optimization and it was found that BChE was quite specific towards chlorpyrifos-oxon in serum with a negligible cross-reactivity towards other CM and OP compounds. The cross-reactivity study was performed using a BChE microplate assay (see section 3.2.3) and the same BChE stock solution was used to prepare the wax-printed strips for the  $\mu$ PAD validation. Notably, the attained CC $\beta$  was  $10 \mu\text{g L}^{-1}$  (Fig. 5) with a false compliant rate equal to zero, as all of the blank ( $n = 20$ ) and spiked ( $n = 20$ ) serum aliquots were correctly classified. It is widely accepted that this is the most important characteristic of a screening method indicating the  $\mu$ PAD potential to assist in the early warning of intoxicated individuals. This success is also related to the standardized illumination achieved by the smartphone-based detection (Fig. 5a).

The screening target concentration (STC) was set to  $10 \mu\text{g L}^{-1}$  as it is significantly lower than OP concentration in real life intoxication incidents [44] (about  $2.5 \mu\text{M}$  or  $870 \mu\text{g L}^{-1}$  chlorpyrifos in serum, for individuals that survived intoxication). Moreover, this STC is suitable for chlorpyrifos-oxon screening based on the formation dynamics of chlorpyrifos to chlorpyrifos-oxon. These dynamics can be either expressed as a ratio of the metabolized chlorpyrifos-oxon concentration divided by chlorpyrifos concentration [45] or as pharmacokinetic models [46]. In the first case, chlorpyrifos-oxon concentration was constantly increased for the first 24 h, reaching about  $80 \text{ nM}$  or  $26 \mu\text{g L}^{-1}$ , a concentration which is more than double compared to the STC. In the latter case, a rate of  $0.35\text{--}0.49 \text{ nM min}^{-1} \text{ mg microsomal protein}^{-1}$  was reported for chlorpyrifos desulfuration to chlorpyrifos-oxon. The aforementioned studies verify the fact that the developed  $\mu$ PAD can be used as an early diagnostic tool for chlorpyrifos intoxication. Worthy to notice is that the developed method achieved better detectability in comparison to other OP screening methods in serum [20,47]. Also, this STC was selected to assure that chlorpyrifos-oxon can be detected after the 40-times dilution of the sample (see 2.9 section). Last but not least, the  $\mu$ PAD device provided also rugged results as validation experiments were performed during 5 different days without significant variation among the responses (Fig. 5b). Importantly, the tested aliquots came from pooled human serum samples indicating the heterogeneity of the tested matrix.

### 3.4. $\mu$ PAD critical comparison towards established methods

Developing a novel analytical method always requires comparing its characteristics towards well-established methods to highlight advantages and drawbacks. Therefore, we critically compared the performance of the developed  $\mu$ PAD towards an in-house BChE microplate

assay and an LC-MS/MS method (Table 2). Among the three methods, the BChE microplate assay achieved the lowest LOD,  $0.033 \mu\text{g L}^{-1}$ , followed by the LC-MS/MS method ( $0.5 \mu\text{g L}^{-1}$ ) and the developed  $\mu$ PAD ( $10 \mu\text{g L}^{-1}$ ). This comes in line to other BChE assays, which achieved sensitive chlorpyrifos-oxon screening [22,48]. Regarding, the LC-MS/MS method (see supplementary materials for method specification) a slightly different sample preparation was applied, in detail, serum aliquots were diluted using an acidified ultrapure water solution (0.1% formic acid v/v). The reason for the aqueous solution acidification was that OPs are more stable in acidic pH [49]. Despite using the least BChE concentration, which is related to the least cost per sample, the microplate assay does not have any portability potential. On the other hand, the  $\mu$ PAD can be potentially applied at the point-of-need achieving a cost  $<0.20 \text{ €}$  per sample. Considering that a LOC injector costs about  $0.30 \text{ €}$  (see section 3.1) and two wax-printed strips are needed, the total cost of the  $\mu$ PAD is about  $0.70 \text{ €}$  at the development stage. Obviously, this cost can be further reduced upon an intensive production. Another unique feature of the  $\mu$ PAD is the minimum expertise needed to operate it as the LOC injector provides integrated sample handling and the smartphone readout a reliable color detection. In fact, the method can be further simplified by developing a smartphone app that would provide instruction on how to apply the test as well as one-click results due to smartphone computing power. In this way, poisoned individuals will be potentially tested using a rapid diagnostic device minimizing the threat

**Table 2**

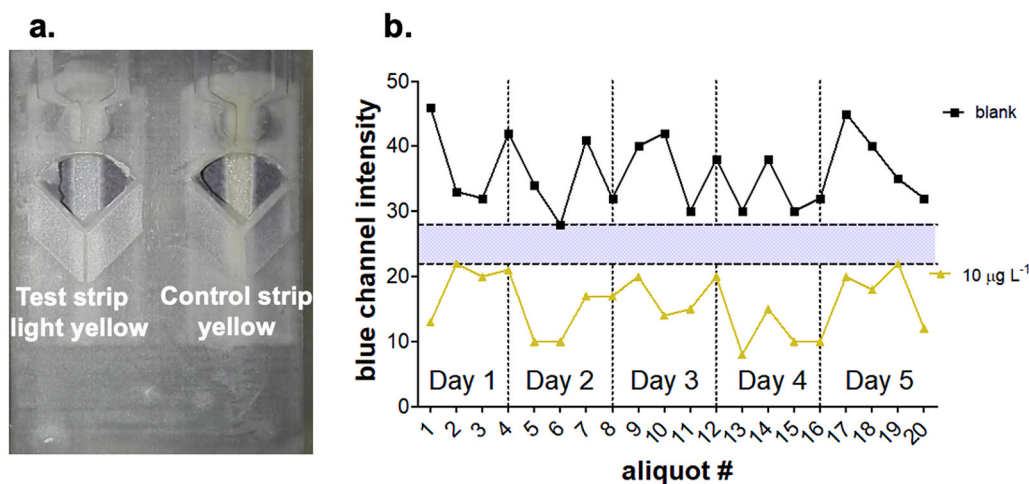
Critical comparison of important characteristics of the  $\mu$ PAD, microplate assay and LC-MS/MS for chlorpyrifos-oxon screening.

Characteristic	$\mu$ PAD	microplate assay	LC-MS/MS
Detectability	<sup>a</sup> $10 \mu\text{g L}^{-1}$	<sup>b</sup> $0.033 \mu\text{g L}^{-1}$	<sup>c</sup> $0.5 \mu\text{g L}^{-1}$
BChE concentration	$0.5 \text{ U strip}^{-1}$	$0.030 \text{ U well}^{-1}$	n.a.
Detection system	smartphone-based color reader	absorbance reader	MS/MS
Sample throughput	two samples/run	up to 96 samples/run	single sample/run
Cost per sample	$\sim 0.16 \text{ €}$	$\sim 0.01 \text{ €}$	$\sim 80 \text{ €}$
Assay duration	10 min	17 min	17 min
Portability	fully portab <sup>a</sup> e	laboratory-based	laboratory-based
Expert <sup>b</sup> se	potentially applicable by non-experts	needed	highly needed

<sup>a</sup> Corresponds to the obtained CC $\beta$  value.

<sup>b</sup> :  $\text{LOD}_{\text{enzyme assay}} = \text{mean}_{\text{blank}} - 3.3 \times \text{SD}_{\text{blank}}$ ,  $n = 6$  blank samples.

<sup>c</sup> :  $\text{LOD}_{\text{LC-MS/MS}} = \text{SD}_{\text{regression}}/\text{slope} \times 3.3$ .



**Fig. 5.** a) A real image of the  $\mu$ PAD assay during a measurement of a control and a test strip at  $10 \mu\text{g L}^{-1}$  chlorpyrifos-oxon; b)  $\mu$ PAD responses of the blank and spiked aliquots to establish CC $\beta$ .

to experience harsh intoxication symptoms.

#### 4. Conclusions

A hybrid  $\mu$ PAD was developed for chlorpyrifos-oxon (a marker of chlorpyrifos exposure) screening in human serum. The developed method combines simplicity (results are related to a color development), cost-effectiveness (0.70 € at the development stage), rapidity (10 min total analysis time) and integrated sample handling due to the use of LOC prototype injectors. Moreover, a smartphone reader was used achieving standardized optical detection. The use of a smartphone as the analytical detector underpins the method potential to provide one-click results by developing a smartphone app. Overall, the  $\mu$ PAD is pocket-size, requires minimal sample preparation (just a sample dilution) and attained robust results. This  $\mu$ PAD can combat chlorpyrifos intoxication incidents by their recognition at an early stage before symptoms are fully expressed.  $\mu$ PAD provides qualitative results and further result verification is necessary by using an instrumental method, e.g. LC-MS/MS. All in all, this method emphasizes the need for point-of-care diagnostics that can assist medical analysis and effectively prevent citizen health.

#### Credit author statement

A.S Tsagkaris: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing. D. Migliorelli: Conceptualization, Methodology. L. Uttl: Investigation, Data curation. D. Filippini: Software. J. Pulkrabova: Project administration, Funding acquisition. J. Hajslova: Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

#### Declaration of competing interest

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

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2020.121535>.

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