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Paper-based colorimetric biosensor of blood alcohol with *in-situ* headspace separation of ethanol from whole blood

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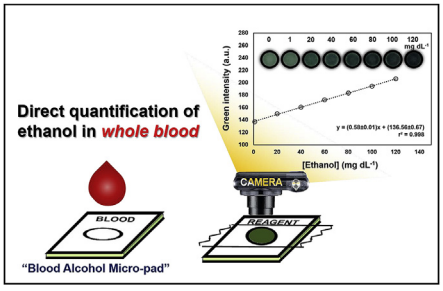
HIGHLIGHTS

- The first paper-based biosensor for quantifying alcohol content directly from whole blood.
- *In-situ* headspace separation with colorimetric enzymatic assay for rapid readout within 5 min.
- The device is portable, low cost and disposable.
- Results are promising for roadside check and toxicological test.

GRAPHICAL ABSTRACT



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This paper is dedicated to Prof. Purnendu (Sandy) Dasgupta on the anniversary of his 70th Birthday (December 5th, 2019)

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ABSTRACT

This work presents a novel development that exploits the concept of *in-situ* gas-separation together with a specific enzymatic colorimetric detection to produce a portable biosensor called "Blood Alcohol Micro-pad" for direct quantitation of ethanol in whole blood. The thin square device (25 mm × 25 mm × 1.8 mm) comprises two layers of patterned filter paper held together with a double-sided mounting tape with an 8-mm circular hole (the headspace). In operation, the reagent is deposited on one layer and covered with sticky tape. Then 8 μL of a blood sample is dispensed onto the opposite layer and covered with sticky tape. Diffusion of ethanol across the 1.6 mm narrow headspace permits selective detection of ethanol by the enzymatic reagents deposited on the opposite layer. This reagent zone contains alcohol oxidase, horseradish peroxidase and 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, as the chromogenic reagent. The color intensity, measured from the recorded digital image, resulting from the enzymatic assay of ethanol, correlates with the concentration of blood alcohol. The results obtained with spiked mice and sheep blood samples, using an external calibration in the range of 1–120 mg dL^{-1}ethanol, gave recoveries of 93.2–104.4% (n = 12). The "Blood Alcohol Micro-pad" gave good precision with %RSD <1 (50 mg dL^{-1} ethanol, n = 10) and limit of quantification (10SD of intercept/slope) of 11.56 mg dL^{-1}. The method was successfully validated against

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a headspace gas chromatography-mass spectrometric method. It has good potential for development as a simple and convenient blood alcohol sensor for on-site testing.

1. Introduction

According to the global status report on road safety by the World Health Organization (WHO) in 2018, 94 countries out of the 175 countries surveyed have data on road traffic deaths arising from consumption of alcohol (ethanol) [1]. To reduce the number of such accidents, each country has passed laws for the maximum blood alcohol concentration (BAC) for a driver. For example, in the United States, United Kingdom and Canada, the legal blood alcohol concentration limits are set at $\leq$ 80 mg dL^{-1}. In Sweden and Russia, the limits are set at < 20 and $\leq$ 30 mg dL^{-1}, respectively [1]. Whereas in most EU countries and some in countries in Southeast Asia including Thailand [1,2] the limit is set at $\leq$ 50 mg dL^{-1}. Due to these laws for measuring driving under the influence (DUI), there is a need for accurate methods to measure "BAC". Measurement of BAC is also an important and frequently requested toxicological test in forensic analysis.

The most widely used method for measurement of BAC is gas chromatography with flame ionization detection (GC-FID) [3–7] or mass spectrometric detection (GC-MS) [6,8,9]. The gold standard method for the determination of blood ethanol is GC-MS with headspace (HS) for *in-situ* separation of ethanol vapor from blood sample prior to analysis by GC-MS [10]. Headspace is employed as it offers direct analysis of volatile components of blood without sample preparation. Quantitation of blood ethanol can also be carried out by using high performance liquid chromatography (HPLC) with fluorimetric detection [11]. Besides chromatographic methods, a number of alternative methods for BAC determination have been developed, *e.g.* horizontal attenuated total reflectance-Fourier transform infrared spectroscopy [12], proton nuclear magnetic resonance (^{1}H NMR) spectroscopy [13] and electrochemical-based biosensors, such as disposable screen-printed electrode modified with Nafion and gold nanoparticles [14]. The classical and low cost method for determination of ethanol in blood utilizes a close system called "Conway diffusion cell" in which a blood sample is placed in the outer chamber of the cell. Alcohol in the blood diffuses *via* a headspace into the central chamber containing a strong oxidant solution, such as acidic potassium dichromate [15]. Heat is required to accelerate the ethanol diffusion and to reduce diffusion time from 10 h (at 25 °C) to 2 h (at 50 °C). The excess dichromate is measured by iodometric titration. Although the method was later improved by replacing the acidic dichromate oxidant with an alkaline potassium permanganate [16], the method still relies on titration and thus not suitable for on-site analysis.

Presently, hand-held alcohol breath analyzer is produced by many manufacturers. It is convenient to use for on-site detection of alcohol in breath and are widely used in many countries for rapid roadside detection of drunk driving. These devices rely on an established ratio between breath alcohol concentration and blood alcohol concentration. However, there are many factors that can influence the analysis by breath analyzers [17,18]. But analysis of alcohol content in blood is still required for confirmation and legal prosecution. In 2018, a portable and reusable microfluidic lab-on-a-chip (LOC) device was developed by Aymerich et al. for direct determination of alcohol in whole blood samples. The biosensor inside the LOC device is an *in-situ* electrodeposition of calcium alginate hydrogel containing enzymes for selective ethanol detection with micro-fabricated platinum electrode [17] and digital display. The device employs a smartphone-based micro-potentiostat. For analysis 40 μL of a blood sample is mixed with the "mediator solution" prior to introducing into the sensing chamber.

For on-site analysis of ethanol, devices using paper substrate are alternative choices to the breath analyzer. In recent years, there have been development of paper-based analytical devices (pads) for determination of ethanol in various matrices [19–25]. These pads have been applied for direct analysis of ethanol in pharmaceutical products [22] and alcoholic beverages [19,20,23]. To the best of our knowledge, paper-based analytical device for direct analysis of blood ethanol has not been reported.

This work presents the development of a paper-based biosensing device named "Blood Alcohol Micro-pad", capable of quantifying ethanol directly from whole blood. The concept of *in-situ* headspace gas-separation (also known as "membraneless gas-separation microfluidic paper-based analytical devices or MBL-GS μPADs) [21] was utilized. This is the first time that ethanol gas is detected using enzymatic colorimetric assay employing paper-based device with *in-situ* headspace gas-separation. The "Blood Alcohol Micro-pad" comprises of two layers of patterned filter paper held together with a double-sided mounting tape with an 8-mm circular hole. The hole provides an *in-situ* thin headspace that allows separation of alcohol vapor from blood sample with selective detection of only the alcohol vapor in a separate detection zone above the headspace. Quantitative measurement of the ethanol vapor employs a reagent comprising alcohol oxidase (AOX), horse radish peroxidase (HRP) and 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the color producing compound. The color intensity is obtained from the recorded digital image using ImageJ software. This design of *in-situ* headspace separation of ethanol, together with the use of enzymatic assay, allows direct analysis of blood samples without the need of pre-treatment. The measurement itself takes 5 min and requires only 8 μL of a blood sample.

2. Material and methods

2.1. Reagents

All chemicals were of analytical grade and solutions were prepared in deionized Milli-Q® water (18.2 MΩ cm). For ethanol analysis, working standard solutions were prepared daily in deionized water by appropriate dilution of 99.9% (v/v) ethanol (Dr. Ehrenstorfer GmbH, Germany). These ethanol standard solutions were kept in tightly sealed containers to prevent loss of ethanol. Two enzymes were used, *i.e.* alcohol oxidase (AOX) (Sigma-Aldrich, USA) and horseradish peroxidase (HRP) (Sigma-Aldrich, USA). The enzyme mixture solution was prepared in 0.5 M of phosphate buffer pH 7.5 to contain 12-unit mL^{-1} of AOX and 30-unit mL^{-1} of HRP, respectively. For the final developed method, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich, Germany) was selected as the color forming reagent. The ABTS reagent was prepared by dissolving 220 mg of ABTS powder in 5.0 mL deionized water. Apart from ABTS, three other chromogenic reagents were also investigated: (i) 50 mM KI

(Merck, USA) prepared in 1% (w/v) starch solution, (ii) aqueous solution of 50 mM of 4-amino-antipyrine (4AAP) (Sigma-Aldrich, Germany) and 50 mM phenol (Sigma-Aldrich, USA) and (iii) a solution of 50 mM of 4AAP and 50 mM of 8-hydroxyquinoline (8-HQ) (Sigma-Aldrich, USA) in 20% (v/v) acetone-water.

2.2. Fabrication of the "Blood Alcohol Micro-pad"

The pad is a square device (25 mm × 25 mm), fabricated by mounting together two layers of ink screen-printed Whatman No. 4 filter paper with a double-sided 1.6 mm thick mounting tape (Scotch™, USA) which has an 8-mm circular disc cut out. Fig. 1a shows a 3D-view of the "Blood Alcohol Micro-pad". In Fig. 1b the side labelled "BLOOD" is where a blood sample is loaded and the side labelled "REAGENT" is where the enzyme reagent is loaded and also where the color image is recorded by the digital camera. Fig. 1c shows the details of the three layers. The printed circular hydrophobic barriers are screen-printed following the method of Sitanurak [22] to delineate the circular reservoirs for the sample and reagent, respectively. The fabrication was adapted from the method

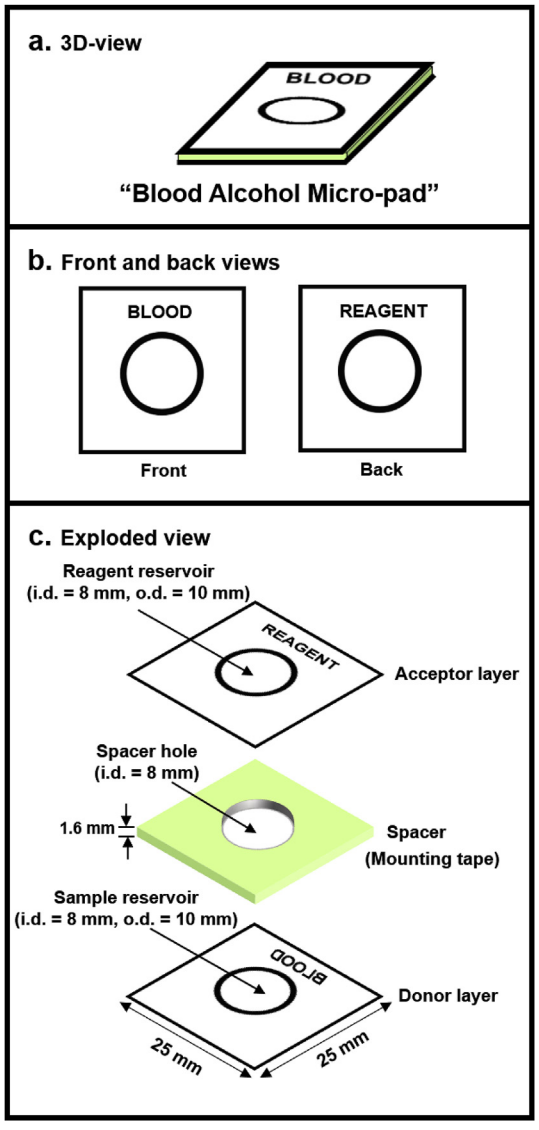

Fig. 1. Schematic drawing of the fabrication of "Blood Alcohol Micro-pad" biosensor for analysis of blood ethanol.

previously described in 2016 [21] with slight modification in the dimensions of the paper device. The reservoir diameter was increased from 6 to 8 mm to increase the area for higher sensitivity.

2.3. Operating procedure

The operating procedure for the determination of blood ethanol using the "Blood Alcohol Micro-pad" is depicted in Fig. 2. In Step 1, 8 μL of 80 mM ABTS reagent (light green solution) is loaded on to the "REAGENT" reservoir. After drying at room temperature for 5 min, 4 μL of the solution of AOX and HRP enzymes is loaded on to the same "REAGENT" reservoir. A piece of transparent tape is then placed over the reagent reservoir (Step 3). In Step 4, 8 μL of blood sample (or ethanol standard) is loaded on the "BLOOD" reservoir. The sample reservoir is then covered with a transparent tape to prevent loss of ethanol vapor (Step 5). Ethanol from the blood sample is allowed to vaporize into the spacer hole to the acceptor zone. The enzymatic reaction with the adsorbed ethanol substrate leads to the change of the color of the ABTS from light green to dark green. Photographic image of the "REAGENT" zone is recorded (using Canon IXUS 125 HS, Japan) at exactly 5.0 min after loading the blood sample. Photography is carried out inside an illuminated box for constant illumination. A red circular paper (6 mm diameter) is placed beside the Micro-pad during the recording of images of the "REAGENT" zone to set the shutter speed and aperture (f-number) at the same values throughout a single batch of measurements. The digital image is analyzed by ImageJ software [26] to give the mean value of the green color intensity of a constant circular area (ImageJ diameter setting of "240") covering the colored part of the "REAGENT" zone. This value is used for calibration or analysis (see Fig. S1 in Appendix A: Supplementary information for details of the photography and analysis of image using ImageJ).

2.4. Sample preparation and analysis

Three samples of mice blood (in 20 mL ampoules) were purchased from National Laboratory Animal Center (NLAC), Mahidol University, Thailand. Three samples of sheep blood (in 20 mL ampoules) were purchased from 'Baan Salaya' Pet Hospital, Nakhon Pathom, Thailand. Five mL of 25,000 I.U. heparin and 400 mg sodium fluoride were added to each blood sample as anticoagulant and preservative, respectively. The samples were analyzed for ethanol content as received and were found to be ethanol-free. To prepare spiked samples, various aliquots of stock solution of 5.00 g dL^{-1} ethanol were spiked into 4.7 mL of the blood sample and the volume made up 5.00 mL with deionized water. These samples were prepared to contain ethanol at 20, 50, 80 and 100 mg dL^{-1}, respectively. The ethanol-spiked blood samples were analyzed in triplicate following the procedure described in Operating procedure Section and by HS-GC-MS, as the reference method. The latter analysis was carried out at an accredited medical laboratory (ISO 15189:2012) of a private hospital in Bangkok (see Appendix B for the operating condition of the HS-GC-MS).

3. Results and discussion

3.1. Selection of color forming agent and reaction time

The colorimetric detection of the "Blood Alcohol Micro-pad" (Fig. 1) utilizes ethanol from the blood sample in the sample reservoir diffusing across the headspace into the reagent reservoir containing reagents that changes color. The color intensity of the reagent reservoir increases with increasing concentration of ethanol in the sample. Preliminary experiments were carried out to select a suitable detecting reagent systems. Four color forming

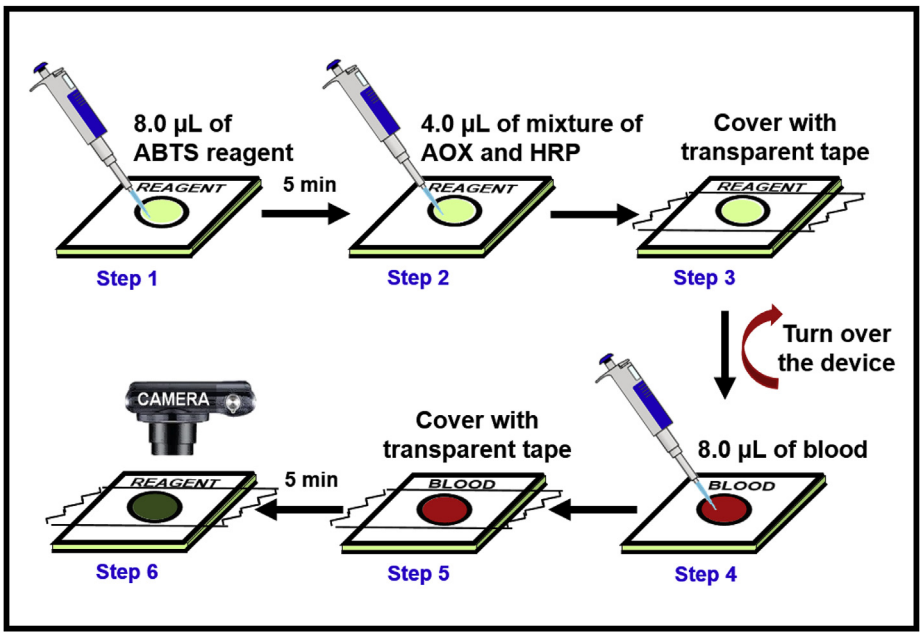

Fig. 2. Operating procedure for determination of ethanol in blood sample using the "Blood Alcohol Micro-pad".

reagents (see Table 1) were tested, including (i) KI + starch reagent [27], (ii) 4AAP + 8-HQ reagent [28], (iii) ABTS reagent [29] and (iv) 4AAP + phenol reagent [30]. All these reagents react with hydrogen peroxide (H_2O_2). Reagents (ii) to (iv) have previously been used to quantitate ethanol by absorption spectrometry. In order to find the suitable color forming reagent for the "Blood Alcohol Micro-pad", each test reagent was loaded onto the "REAGENT" reservoir, followed by the enzyme solution. Standard ethanol solution was loaded on to the "BLOOD" reservoir, in the same manner as described in Operating procedure section. (Details of the operations are shown in Fig. S2 in Appendix B). The adsorbed ethanol vapor is enzymatically converted to H_2O_2 by AOX in the reagent reservoir. The H_2O_2 then oxidizes the color forming reagent catalyzed by the HRP enzyme.

Kinetic studies of all the four reagents were carried out using two ethanol standard solutions of 50 and 100 mg dL^{-1}, respectively. Fig. S3 (in Appendix B) shows the plots between the color intensity obtained from the image of reagent reservoir (Step 6, Fig. S2) and reaction time, *i.e.* the time interval between loading of ethanol sample (Step 4, Fig. S2) and the recording of the image (Step 6, Fig. S2). The kinetics were followed over reaction times of 1–15 min to determine the optimum reaction time for each detection reagent. For the "KI + starch reagent" (Fig. S3a), a maximum color intensity was achieved after 3.5 min. However, the color intensity then continuously decreased due to the instability of the tri-iodide-starch complex. The reaction time of 3.5 min was chosen for this reagent in the further study (see below). As for the "4AAP + 8-HQ", "ABTS" and "4AAP + phenol" reagents (Fig. S3b – S3d), it was observed that the color intensity increased with reaction time, reaching a maximum value which remained constant. The reaction time required to reach this maximum intensity for each of these three reagents were 4, 4 and 11 min, respectively.

All four reagent systems were used to construct calibration plots employing the "Blood Alcohol Micro-pads". Linear calibrations were obtained from all reagents with coefficient of determination (r^2) very close to 1 (see Table 1). However, the reaction time of the "4AAP + phenol reagent" is relatively long (11 min) and this reagent was therefore not selected. The "KI + starch" reagent was also not chosen since the color of the starch complex is not stable. Comparing the "4AAP + 8-HQ" reagent with the "ABTS" reagent, the sensitivity of both reagents are comparable (see Table 1). However, the preparation of 8-HQ solution requires the use of acetone which is not compatible with hydrophobic barriers of the "Blood Alcohol Micro-pad". Thus in the further development of the device, ABTS was selected as the color forming reagent.

3.2. Sample volume and condition of the ABTS reagent

In the operating procedure (Fig. 2), a transparent tape is placed over the sample reservoir immediately after sample loading to prevent loss of ethanol vapor. The volume of sample must be totally absorbed into the pores of the paper of the sample reservoir. Excessive amount of sample will spill over the hydrophobic barrier giving irreproducible results. In preliminary tests using a color dye

Table 1
Calibration equations obtained from loading a series of standard solutions of aqueous ethanol (50–100 mg dL^{-1}) on the "Blood Alcohol Micro-pads" using the four systems of chromogenic reagent.

Color forming reagent	Reaction time (min)	Calibration equation	r^2
(i) KI + starch	3.5	$y = (0.378 \pm 0.019)x + (90.9 \pm 1.2)$	0.99
(ii) 4 AAP + 8-HQ	4	$y = (0.447 \pm 0.092)x + (101.1 \pm 6.0)$	0.96
(iii) ABTS	4	$y = (0.559 \pm 0.065)x + (110.8 \pm 4.2)$	0.99
(iv) 4 AAP + phenol	11	$y = (0.677 \pm 0.044)x + (95.7 \pm 2.8)$	0.99

y: green intensity.
x: ethanol concentration in mg dL^{-1}.

solution and loading with 2–15 μL, it was observed that the circular reservoir (8 mm i.d.) could accommodate solution up to a maximum volume of 10 μL before the dye was seen outside the circular reservoir. Volumes less than 8 μL did not completely cover the reservoir surface. Similar results were observed when testing with mice and sheep bloods. Therefore 8 μL was chosen as the most suitable sample volume.

Since the circular reagent reservoir for loading the ABTS reagent has the same diameter as the circular sample reservoir, the volume of 8 μL was also employed for loading the ABTS solution. The concentration of ABTS was also investigated at 40, 60, 80 and 100 mM, respectively. Calibration graphs, using standard ethanol solutions (20–100 mg dL^{-1}) were constructed for each concentration of ABTS. The slopes and the intercepts were found to be comparable (data not shown). However, the coefficient of determination (r^2) for 40 mM ABTS (r^2 = 0.94) and 60 mM ABTS (r^2 = 0.97) were slightly lower than the value for 80 mM ABTS (r^2 = 0.98) and 100 mM ABTS (r^2 = 0.98). For this work, ABTS concentration at 80 mM was therefore selected as the operating concentration.

3.3. Analysis using "Blood Alcohol Micro-pad"

3.3.1. Calibration and linear range

The linear working range of the "Blood Alcohol Micro-pad" was investigated employing the protocol shown in Fig. 2 using aqueous ethanol standard solutions from 1 to 200 mg dL^{-1}, respectively. Values of the green color intensity of these images (obtained using ImageJ) were employed to give the calibration plots as shown in Fig. 3a. It can be seen that the linear region extends only to the concentration of 120 mg dL^{-1} ethanol. Thus the linear working range is between 1 and 120 mg dL^{-1} ethanol.

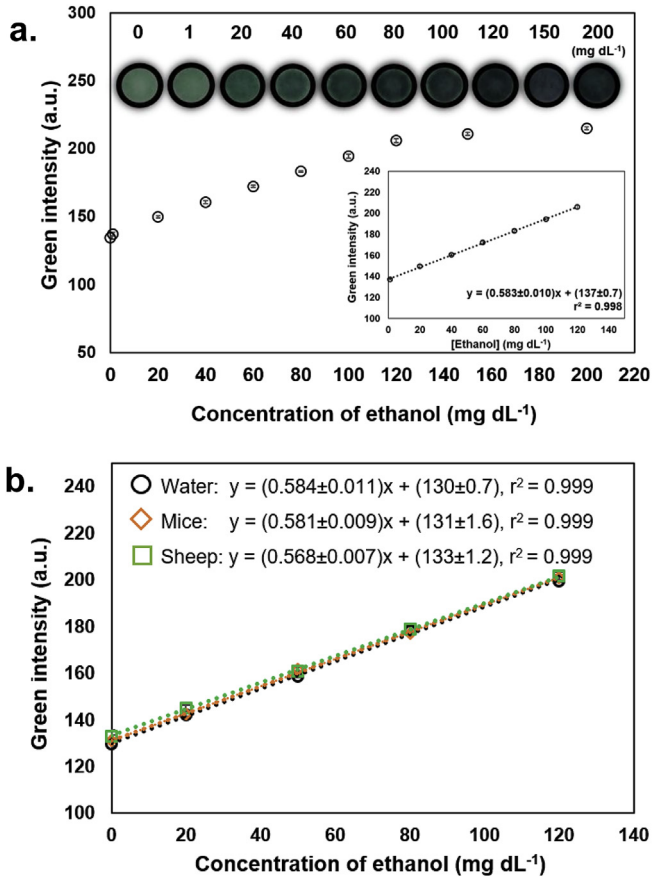

Fig. 3. (a) Plot of the green intensities for various concentrations of aqueous ethanol and (b) calibration graphs of ethanol in water, mice blood and sheep blood using the "Blood Alcohol Micro-pad": y is green intensity, x is ethanol concentration in mg dL^{-1}. Inset shows the color for each of the ethanol samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3.2. Validation of use of external calibration for blood alcohol determination

Three sets of experiment were carried out using the "Blood Alcohol Micro-pad" to test the validity of using an external calibration. The procedure in Fig. 2 was employed in these experiments. A calibration graph using a series of aqueous standard ethanol solutions, 20–120 mg dL^{-1}, was first constructed. Two other calibration graphs using spiked mice and sheep blood were constructed over the same concentration range of ethanol. As shown in Fig. 3b, all three lines are visually superimposed; they have the same values of the slopes and intercepts. The results confirm that external calibration method is valid for use with the "Blood Alcohol Micro-pad".

3.3.3. Analytical features

The calibration is linear from 1 to 120 mg dL^{-1} with a typical calibration equation: Green intensity = (0.583 ± 0.010) × mg dL^{-1} EtOH + (137 ± 7): r^2 = 0.998 (see Fig. 3a). The limit of quantification (LOQ), defined as LOQ = 10 × SD of intercept/calibration slope, is 11.56 mg dL^{-1}. The method provides good precision with RSD less than 1% (for 50 mg dL^{-1} ethanol, n = 10). With these features, the biosensor shows good potential for future application with human blood since the linear working range covers the concentrations of blood ethanol found in the samples collected from drunken motorists [31]. The LOQ value of the "Blood Alcohol Micro-pad" of 11.56 mg dL^{-1} is also lower than the limits of illegal level of blood ethanol in many countries, including USA, Sweden, Russia and Thailand (see Introduction) [1].

3.3.4. Applications and validation

The "Blood Alcohol Micro-pad" biosensors, using the protocol shown in Fig. 2, were employed for direct analysis of 3 mice and 3 sheep blood samples. HS-GC-MS was employed as the reference method to validate our "Blood Alcohol Micro-pad". Since all animal blood samples were ethanol-free samples, the measured ethanol contents were below the detection limit using the pad biosensor and the HS-GC-MS methods. In order to validate the biosensor, standard ethanol was spiked into these blood samples to contain ethanol at 20, 50, 80 or 100 mg dL^{-1}, as shown in Table 2. The results in Table 2 shows good agreement between our biosensor method and the HS-GC-MS method as shown by the paired *t*-test (t_{stat} = 0.501 and t_{crit} = 2.201: P = 0.05). The Pearson plots (Fig. S4) also confirm the good agreement between the two methods, with slope of 1.00 ± 0.02, intercept of 0 ± 3, and r = 0.999, for both mice (Fig. S4a) and sheep bloods (Fig. S4b).

3.4. Comparison with other methods

Table 3 compares the features of five methods for quantifying blood alcohol content including this method. Comparing between the two HS-GC-MS methods, the method of Xiao et al. [9] has some advantages over the method of Heit et al. [8] in terms of not requiring sample pre-treatment, better precisions and significantly shorter analysis time (5 min compared to 20 min). However, the method of Xiao et al. [9] has the lowest LOQ amongst the five methods in Table 3. The traditional microdiffusion method is laborious with long analysis time from 2 h (with heating at 50 °C) to 10 h (at 25 °C). The disposable biosensor [14] offers a very rapid

Table 2
Validation data of blood ethanol quantification.

Sample	Spiked ethanol (mg dL^{-1})	Blood Alcohol Micro-pad		HS-GC-MS	
		[EtOH] (mg dL^{-1})(n = 3)	%Recovery	[EtOH] (mg dL^{-1})(n = 3)	%Recovery
Sample 1	20	20.39 ± 1.52	93.2	20.08 ± 0.51	100.2
	50	52.71 ± 1.84	101.9	51.64 ± 0.97	103.2
Sample 2	50	51.19 ± 2.10	98.9	51.16 ± 1.63	102.2
	80	82.34 ± 1.95	100.7	84.30 ± 2.65	105.3
Sample 3	80	81.98 ± 1.79	100.3	81.80 ± 0.45	102.2
	100	98.03 ± 1.56	96.3	98.58 ± 2.92	98.4
Sample 4	20	21.41 ± 2.13	100.3	18.58 ± 0.20	98.5
	50	52.56 ± 1.60	104.7	52.01 ± 1.37	103.9
Sample 5	50	50.76 ± 0.61	99.5	50.07 ± 0.39	100.0
	80	84.16 ± 1.14	104.4	82.04 ± 0.43	102.5
Sample 6	80	82.44 ± 1.81	101.4	83.75 ± 1.26	104.6
	100	99.54 ± 1.31	98.2	101.08 ± 1.14	101.0

Samples 1–3 are mice bloods and samples 4–6 are sheep bloods.

Table 3
List of methods that have been developed for blood ethanol analysis.

Method	Sample		Linear range (mg dL^{-1})	LOQ (mg dL^{-1})	%RSD	Analysis time
	Pretreatment	Volume (μL)				
1. HS-GC-MS						
1.1 Xiao et al. [9]	Not required	1000	4–126	4	0.7–3.8 (n = 6)	5 min
1.2 Heit et al. [8]	Centrifugation[a]	300	0.05–460	0.046	4.3–8.8 (n = 5)	20 min[b]
2. Microdiffusion + titration [15]	Not required	250 -1000	n.a.	n.a.	n.a.	2 h (50 °c)
3. Disposable biosensor based on screen-printed electrode[c] [14]	Centrifugation[s] and dilution	n.a.	0–36.8	n.a.	6.98 (n = 10)	40 s
4. Reusable-electrochemical Lab-on-chip biosensor platform [17]	Not required	40	0–125	12.5	~4.7–7[d] (n = 6)	2 min
5."Blood Alcohol Micro-pad" (This work)	Not required	8	1–120	11.6	<1 (n = 10)	5 min

n.a.: not available.
[a] Pretreatment by centrifugation to obtained serum from whole blood.
[b] Excluding centrifugation time.
[c] With use of separate saturated calomel reference electrode and platinum wire counter electrode.
[d] Estimated from the data (the calibration plot).

analysis time of 40 s but requires two sample preparation steps to obtain diluted serum prior to the amperometric detection. The reusable-electrochemical lab-on-a-chip (LOC) biosensor platform [17] and our biosensor pad offer comparable features for the linear working range and the LOQ. Our method is slower than the LOC biosensor method (5 min compared to 3 min). However, the LOC platform needs time between samples to clean the fluidic channel and the electrodes to prevent cross contamination. The "Blood Alcohol Micro-pad" is a single use device. The very low %RSD (<1% for 50 mg dL^{-1} ethanol, n = 10 pads) confirms that the production of "Blood Alcohol Micro-pad" is reproducible. This precision is as good as the HS-GC-MS method [9]. Our method requires the least volume of sample (8 μL) amongst all the other methods in Table 3. The linear range of our biosensor covers the linear range of the gold standard HS-GC-MS method [9]. Although the LOQ of our method is approximately 3 times larger than the HS-GC-MS method, the method is fit for the purpose of blood alcohol analysis [1].

4. Conclusions

To the best of our knowledge, the "Blood Alcohol Micro-pad" is the first paper-based biosensor that employs enzymatic colorimetric assay with *in-situ* headspace gas separation for direct determination of ethanol from whole blood. The device is very small in size (25 mm × 25 mm × 1.8 mm), light in weight (*ca* 0.5 g) and disposable. It is fabricated from filter paper and mounting tape. Screen-printing technique is employed to print hydrophobic barrier on the filter paper to define areas for applying the blood sample and the detecting reagents. Production costs is very low (at *ca.* 7USD/100 devices). The selectivity of the method is ensured by employing two processes: (i) ethanol vapor is first separated from the blood matrix *via* diffusion through the *in-situ* headspace that connects the sample compartment and detection zone and (ii) specific detection of ethanol employing the use of two enzymes, *i.e.* AOX and HRP. The AOX enzyme employs ethanol as substrate for production of acetaldehyde and hydrogen peroxide [32]. The chromogenic ABTS reagent reacts with the generated hydrogen peroxide, catalyzed by HRP, in the detection zone to give a dark green color. Analysis of the digital image of the "REAGENT" zone using ImageJ software provides color intensity values. The precisions of the "Blood Alcohol Micro-pad" is comparable with the gold standard HS-GC-MS method [9] but using much lower volume of blood sample (8 μL comparing to 1 mL). Blood collection can be obtained using the blood sampling tool used for daily glucose analysis for diabetic patients. We believe that this biosensor pad has potential for development as a roadside test for drinking drivers, as well as bed-side test in hospital or clinic.

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.

CRediT authorship contribution statement

Yanisa Thepchuay: Conceptualization, Methodology, Investigation, Writing - original draft. **Thitaporn Sonsa-ard:** Formal analysis, Visualization. **Nuanlaor Ratanawimarnwong:** Validation, Resources. **Saranya Auparakkitanon:** Formal analysis. **Jirayu Sitanurak:** Conceptualization, Methodology, Data curation. **Duangjai Nacapricha:** Conceptualization, Supervision, Project administration, Writing - review & editing, Funding acquisition.

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This paper is dedicated to Prof. Purnendu (Sandy) Dasgupta on the anniversary of his 70th Birthday (December 5th, 2019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.12.043.

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