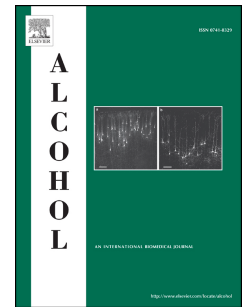


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AWARE: A Wearable Awareness with Real-time Exposure, for monitoring alcohol consumption impact through Ethyl Glucuronide detection.

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Abstract

Here we demonstrate for the first time a dynamic monitoring of the ethanol metabolite Ethyl Glucuronide (EtG) for a more robust evaluation of alcohol consumption compared to conventional methods. A wearable biosensor device capable of reporting EtG levels in sweat both continuously via low power impedance spectroscopy is reported. The custom hardware was compared against conventional benchtop potentiostat, demonstrating comparable results in the application space of EtG detection in low volume sweat. The device successfully differentiated three distinct EtG concentrations correlating to simulated drinking scenarios estimated to be 1, 2, and 3 standard U.S. drinks consumed over a duration of 60 minutes with $p < 0.0001$. This device has the potential to enable moderate drinkers to engage in guided decision making, based on objective data, addressing the needs of alcohol sensitive populations. The device also will serve as a tool for researchers to better understand and characterize the relationship between sweat EtG and consumed alcohol.

Highlights:

- **Demonstration of a stand-alone wearable biosensor for non-invasive monitoring of sweat EtG.**
- **Detection of EtG continuously towards real-time alcohol consumption monitoring.**
- **Comparable performance with benchtop potentiostat.**
- **Differentiation of EtG concentrations estimated to correlate to 1 – 3 consumed drinks.**

Keywords:

Wearable, biosensor, Ethyl Glucuronide (EtG), sweat, electrochemical impedance spectroscopy (EIS), continuous sensing

Introduction

According to the 2015 National Survey on Drug Use and Health (NSDUH) 56% of people aged 18 years or age and older stated they had consumed at least one alcoholic within a month's time. In that duration, 27% of the same demographic reported engaging in binge drinking (4+ drinks for women and 5+ drinks or men). It stands to reason that moderate drinking (1 drink per day for women and 2 drinks per day for men), falls between those percentages highlighting its proclivity in U.S. society. Moderate drinking, though prevalent, does come with a host of concerns that warrants the personal monitoring of these events: physical risks can include direct consequences such as motor vehicle accident during the period of intoxication as well as influence on personal health such as increased risk of stroke, cancer, negative interactions with medication, impact on pregnant mother's, or mothers who are nursing (NIAAA, 1992). To date, alcohol consumption monitoring devices typically report blood alcohol content (BAC) through correlation with either breath alcohol content (BrAC), or transdermal alcohol content

(TAC) (Ashdown, Fleming, Spencer, Thompson, & Stevens, 2014; Leffingwell et al., 2013). The rapid expression of ethanol in breath and sweat is beneficial for intoxication monitoring. However, the rapid decay of expressed ethanol in those medias can equally limit the application space of these devices.

Alcohol is metabolized and excreted from the body by different processes or pathways. (Kelly & Mozayani, 2012)

- (1) Direct excretion of alcohol (5-10%) in urine, sweat and breath;
- (2) Metabolic excretion to acetaldehyde and quickly broken down to acetate (>90%);
- (3) Metabolic excretion by conversion to ethyl Glucuronide (EtG) and ethyl sulfate (EtS), which is produced in the liver through the process of glucuronidation.

EtG and EtS represents a low total disposition of ethanol (~0.1%) and is readily expressed and detectable in transdermal human sweat (Cederbaum, 2012; Ramchandani, Bosron, & Li, 2001; Schummer, Appenzeller, & Wennig, 2008) making it a promising candidate for wearable alcohol consumption monitoring, as shown previously by our group (Kinnamon, Muthukumar, Selvam, & Prasad, 2017; Panneer Selvam, Muthukumar, Kamakoti, & Prasad, 2016). Most of these excretory biomarkers are detectable in urine for short period of time (~24hrs). EtG, with its longer half-life, can be detectable in urine for up to 130 hours for heavy drinkers (Helander, Bottcher, Fehr, Dahmen, & Beck, 2009). EtG is also excreted in blood/urine around 60 minutes after alcohol consumption. Figure 1B shows the typical time course for ethanol and EtG expression in blood (Hoiseth et al., 2007), which is highly correlated with expression in sweat. Here we propose that as an ethanol metabolite, EtG can potentially serve as an indirect indicator of alcohol consumption and metabolism with, compared to other direct markers, a prolonged detection window. Such a sensor would provide researchers a point-of-use tool to investigate with more precision the long-term impact of even light to moderate drinking in susceptible populations non-invasively. Currently, there is not a robust tool for evaluating sweat EtG in these populations. The development of an EtG-based non-invasive biosensor may provide a long-standing quantitative of ethanol metabolism and its potentially harmful metabolites after the initial peaks of ethanol expression have subsided, which can improve the ability for susceptible populations to socially drink safely while improving researcher's understanding of alcohol metabolites impact on these populations.

With this vision in mind, we are demonstrating for the very first time, a wearable biosensor prototype that has the potential to quantitatively monitor of transdermal EtG. This device, also known as AWARE has demonstrated the ability to detect cortisol in sweat and was adapted for EtG sensing (Sankhala, Muthukumar, & Prasad, 2018). The device uses electrochemical impedance spectroscopy (EIS) and antibody-based affinity capture assay built upon a disposable custom porous polymer test strip to quantify expression of EtG in human sweat. When a single alcoholic beverage (~14 g of pure ethanol (CDC, 2017)) is consumed, EtG will be expressed in sweat at an effective

concentration in a given time (Schummer et al., 2008). We want to study the relationship between the EtG expressed in sweat with respect to the number of drinks consumed in a given time. However, there are many factors will affect these results, such as age, gender, weight/height, fitness, genetics and other factors such as proximity of last meal that can all drastically modulate the rate of alcohol metabolism and impact how long it remains in your system after drinking (NIAAA, 1997; Zakhari, 2006). Before we take these factors into consideration, we have to make sure our wearable biosensor prototype can distinguish different number of drinks on bench. To do this, three different concentration ranges of EtG, based on single alcoholic beverage in one hour, have been designed in order to mimic common drink scenarios during moderate drinking (~14 g of pure ethanol (CDC, 2017)). Here, three different concentration ranges of EtG expression were evaluated: 13.3, 26.7, and 40 ng/mL which, based on literature, are predicted to correlate to between 1 – 3 standard U.S. drinks (Høiseth et al., 2010; Jatlow et al., 2014; Schummer et al., 2008).

Methods

Materials and Reagents

The linker molecule Dithiobis [Succinimidyl Propionate] (DSP) and its solvent Dimethyl Sulfoxide (DMSO) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Synthetic sweat composition prepared in the laboratory contains uric acid, lactic acid, ammonia, Na^+ , K^+ and Cl^- ions. The composition for synthetic sweat was adopted from Mathew et al. (Mathew, Ariza, Rocha, Fernandes, & Vaz, 2008). Phosphate Buffered Saline (PBS), and non-BSA tagged Ethyl- β -D-glucuronide (EtG) were purchased from Sigma Aldrich (St. Louis, MO), and monoclonal EtG Antibody was purchased from EastCoast Bio (North Berwick, ME).

Sensor Fabrication

Polymer substrates were patterned with conductive electrodes using a two-step shadow mask deposition. Shadow masks were laser cut to specified pattern using a Spirit Spectra-Physics laser manufacturing system. The electrode stack comprised of an initial semi-conductive ~125 nm layer of ZnO thin film pattern deposited using radio frequency magnetron sputtering. ZnO was deposited at 140 watts for 60 minutes using 12 sccm argon flow. 12 nm of conductive gold electrodes were patterned upon the ZnO layer. The gold on the ZnO sensor forms an ohmic contact to the ZnO film and the potentiostat.

AWARE device design

The Aware device, shown in its wearable form-factor in Figure 1A is adapted from the device shown in Sankhala et al. (Sankhala et al., 2018). This device consists of four subsystems. Analog Devices ADuCM350 system-on-chip (SoC) was used to implement an impedance measurement loop and communicate with the other three sub systems. The Bluetooth communication was established using the Nordic nRF8001 Bluetooth chipset. This chipset allows for reporting and storing of data with a companion app on a smartphone or on a computer or smartphone, but for simplicity the data was extracted via

USB for this study. Texas Instruments HDC1080 was used to sense the environmental temperature and relative humidity. Texas Instruments BQ24040 was used to implement a USB/Li-ion battery power management system. The SoC consists of a configurable analog front end (AFE) which consists of a wave generator, a switch matrix and a 2048-point discrete Fourier transform based impedance analyzer. Two analog pins were selected from which one acts as a generator and one acts as a sink. These pins are in turn connected to one of the sensors due to the action of the switch matrix. After a 13 millisecond DFT cycle on one sensor, the AFE switches to the next sensor channel. Thus, impedance measurement was performed on all four sensors in a combinatorial sequence. In benchtop testing, this functionality was used to produce the multiple replicates, while in practice it will be used for redundancy. A photograph of the sensor affixed to a user is presented in Figure 1A.

Affinity assay functionalization and protocols

Thiol-linker functionalization was carried out by dispensing 3 μL volume of 10 mM DSP diluted in DMSO onto the ZnO sensing area of each tested sensor. Each sensor was incubated at room temperature for three hours in dark, followed by 30 minute incubation of 100 $\mu\text{g/mL}$ of anti-EtG monoclonal antibodies diluted in PBS at room temperature. After successful functionalization of antibody, 3 μL volume of synthetic sweat solution was dispensed onto the sensor strip prior to an EIS measurement. For calibration dosing responses 3 μL of EtG doses (1, 10, 100, 1000, 10000 ng/mL) prepared in synthetic sweat were added to the sensing region and allowed to incubate for 5 minutes before EIS measurement was taken. Subsequent increasing concentrations were serially added and incubated following the same procedure to produce the calibration dose response. The described protocol is adapted from our group's previously published ZnO cortisol biosensor work, which was performed on a similar substrate (Munje, Muthukumar, Panneer Selvam, & Prasad, 2015). Volumes for the functionalization steps were maintained at or below 5 μL due to the effective volume of the sensor. EIS measurements were taken either by benchtop potentiostat (Gamry Instruments, Warminster, PA, USA) or on the wearable AWARE platform. A low amplitude sinusoidal waveform (10 Hz to 1 kHz) was used to conduct impedance measurements on both platforms. The impedance in all experiments was analyzed at 200 Hz, where a maximized 60 degrees phase lag in the output impedance response was observed indicating the capacitive nature of the sensor response.

Design drinking scenarios

In order to test the AWARE device in a more practical manner, sensors were continuously dosed in regular intervals with fixed concentrations of EtG in synthetic sweat that accurately correlate to the projected EtG composition of a person after consuming 1, 2, or 3 drinks in a single hour. Published studies on quantitative EtG measurements demonstrated that collected sweat from human volunteers using a PharmChek patch produced 0.0017 to 0.103 $\mu\text{g/mL}$ of EtG when 38.0 to 154.6 grams of

equivalent pure ethanol was consumed, when analyzed using mass spectrometry (Schummer et al., 2008). According to the Centers for Disease Control and Prevention, USA, a standard drink in US is equal to 14.0 grams (0.6 ounces) of pure alcohol (CDC, 2017). Based on the relationship between sweat EtG and ethanol in Schummer et al it was calculated that a standard 14 g drink would be equivalent to approximately 24 ng/mL/hr of EtG in sweat scaling proportionally with increased number of drinks by linearizing the results. The EtG levels to consumed ethanol is an assumption supported by behaviors observed by Jatlow et al. (Jatlow et al., 2014). Based on the form factor of the sensor, the maximum volume the sensor can hold is about 20 μ L. To get final a concentration of EtG to 24 ng/mL on the sensor within a one-hour window of time, the total amount of EtG applied on the sensor should be 0.48 ng. This can be realized by continuously dosing 3 μ L of EtG 12 times over the course of the hour at an effective concentration of 13.3 ng/mL of EtG in sweat. This relationship scales linearly with respect to number of drinks. So, 26.7 and 40.0 ng/mL were used to simulate 2 and 3 drinks in a given hour receptively.

The sensors were tested at room temperature (23–25 °C) and ambient relative humidity (~45%). The experiment was broken into three regimes. In the first regime, five replicates of EtG-lacking synthetic sweat was applied at a rate of 3 μ L every 5 minutes to the sensor in order to stabilize the EIS response. In the second regime, 12 applications of 13.3 ng/mL EtG were applied at a rate of 3 μ L every 5 minutes. In the last regime, another five replicates of EtG-lacking synthetic sweat were applied on the sensor at the same rate to represent stoppage of drinking. The same protocol was followed for 2 and 3 estimated drinks but at 26.7 and 40 ng/mL concentrations in the second regime respectively.

Results and Discussion

EIS calibration of EtG detection in synthetic sweat.

Non-Faradaic EIS measurements were used to quantify the binding interactions of EtG antigen in synthetic human sweat to the EtG-specific immunoassay constructed upon the polymer sensor. Binding was transduced based on the capacitive changes that occur at the electrical double layer (EDL) using the non-faradaic EIS technique. We have previously demonstrated this modality of sensing for single capture affinity assays in a number of supporting publications (Kinnamon, Ghanta, Lin, Muthukumar, & Prasad, 2017; Kinnamon, Muthukumar, et al., 2017; Munje et al., 2015; Munje, Muthukumar, & Prasad, 2017; Shanmugam, Muthukumar, Selvam, & Prasad, 2016). A schematic for this constructed EtG detection assay is presented in Figure 1C. The resulting binding of EtG and modulation of the EDL is transduced by the resulting change in effective impedance of the sensor. Figure 2 shows the percent of change in impedance of the sensor with respect to various dose concentrations of EtG in synthetic human sweat captured at 200 Hz collected on both the benchtop instrument and AWARE platform. On the benchtop platform, the sensors demonstrated detection of EtG with signal above the noise in the range of 10 – 10000 ng/L. There is a consistent change in impedance as the dosage

concentration of EtG increases, manifesting as: 20.8 % $\pm$ 1.97 % for 10 ng/mL, 30.4 % $\pm$ 1.39 % for 100 ng/mL, 34.8 % $\pm$ 1.51 % for 1000 ng/mL, and 37.7 % $\pm$ 1.27 % for 10000 ng/mL. The error bars are standard error of mean from at least 6 measurements per data point. The governing equation for the impedance response with respect to EtG concentration on the benchtop potentiostat can be described as:

$$\text{Change in impedance (\%)} = 2.42 * \ln(\text{EtG}_{\text{ng/mL}}) + 17.12 \quad (1)$$

The performance of the benchtop potentiostat was used as a point of comparison for the performance of the AWARE wearable biosensor device. The same protocol was tested on the AWARE platform at 200 Hz to demonstrate proof of translatability of the proposed EtG biosensor onto an economical and compact form factor. Figure 2 also shows the percent change in impedance from the baseline measurement for the wearable device. The percent change with respect to baseline measurement was observed to be: 20.0 % $\pm$ 0.62 % for 10 ng/mL, 27.5 % $\pm$ 0.48 % for 100 ng/mL, 31.8 % $\pm$ 0.48% for 1000 ng/mL, and 35.9 % $\pm$ 0.25 % for 10000 ng/mL. The error bars are standard error of mean from at least 6 measurements per data point. The governing equation for the impedance response with respect to EtG concentration on the AWARE wearable device can be described as:

$$\text{Change in impedance (\%)} = 2.51 * \ln(\text{EtG}_{\text{ng/mL}}) + 13.57 \quad (2)$$

When compared to the previous response on the benchtop instrument, these results show that the AWARE device is performing comparably to the conventional benchtop instrument exhibiting a negligible difference in logarithmic slope, and a modest and predictable $\sim$ 3.5% offset difference in impedance. These results demonstrate that the AWARE device can be used with confidence in future studies as an accurate reporter of impedance change with respect to EtG concentrations.

Continuous detection of EtG in three drinking scenarios

Now that the sensor has been validated for EtG detection, the AWARE device was tested in a more practical manner, simulating its ability to distinguish three different simulated drink scenarios from one another to motivate the technology's potential ability to quantitatively report through sweat EtG, an indirect estimate of ethanol metabolism. Figure 3A and Table 1 depict the continuous dosing of EtG to replicate the projected three drinking scenarios of 1 - 3 drinks per hour. With further deployment of our device we may find that the relationship between EtG concentration and effective consumed alcohol. However, the basis of this work is to demonstrate the ability to quantify and differentiate various EtG dosages in a wearable form-factor, it was assumed based on cited literature that the relationship between EtG and consumed ethanol is a linear one at lower consumption levels (1 – 3 drinks) and that tested levels are in the physiologically relevant range even if they may be adjusted slightly in terms of the absolute number of drinks they correlate to.

Figure 3B depicts the continuous response of EtG for the three different estimated drink profiles. Change in total impedance was calculated at 200 Hz with respect to baseline for all dose additions and was performed in triplicate. The results indicate that by the end of the first regime a total change in impedance of 359 Ω , 619 Ω , and 630 Ω was observed for 13.3, 26.7, and 40 ng/mL plots before the addition of EtG. In the second regime, at the end of EtG dose application, the total change in impedance was 3458 Ω , 4327 Ω , and 5172 Ω for 13.3, 26.7, and 40.0 ng/mL of EtG respectively. This shows that the sensor is relatively unresponsive to the addition of sample without the presence of EtG. The changes in impedance only came from the stabilization of the sensor. The sensor only responds in a discernable way once EtG containing sample is applied. Even more, the change in impedance corresponds with the estimated drink scenarios, increasing in magnitude of response with higher number of drinks. These results show that the sensor is responding to EtG binding at the electrode interface and is not responding to fluid loading on the device, which is an important distinction in electrochemical wearables. In the third and final regime, the same protocol as the first regime was followed, applying EtG-lacking sample. All three tested scenarios stop responding once EtG is no longer present in the test sample increasing by only 134 Ω , 127 Ω , and 74 Ω from the previous regime for 13.3, 26.7, and 40.0 ng/mL respectively over the course of five additions. This reinforces that the sensor is only responding to the EtG present in the applied samples. Using an unpaired t-test to compare the five measurements from regime three from each scenario with one another, a p-value of less than 0.0001 was realized for all comparisons, demonstrating statistical significance and motivating differentiation of the conditions. Though, it is evident at the end of the experiment that each test scenario distinguishes itself from the others in a linear manner as evidenced by the increasing plateau in impedance response in Figure 3B, it is important to understand the dynamics of EtG expression in real-time to give a more immediate feedback to the wearer, not only at the end of day. Figure 3D represents the rate of change in impedance as a function of time for the data in Figure 3B. The rate of change responds in a way that correlates well with the dosing response in Figure 3B, where the three responses begin to differentiate only as dosing occurs before returning back to the initial response as dosing ceases. Though it is clear based on the lesser degree of separation at the initial dosing steps that there will be a modest delay in the sensor's ability to differentiate EtG concentrations at a given time relative to what is physically present on the sensor, these results demonstrate an invaluable first step in achieving real-time detection of EtG in sweat. The implementation of analytical techniques may prove to predict current EtG expression levels based on the real-time sensor response compensating for this delay.

Proposed implementation

The AWARE biosensor has the potential to become a commercial device for at-risk populations to better monitor the effects of their alcohol consumption, more precisely, before engaging in potentially harmful activities such as premature administration of medication. However, there is still much to be learned about the expression of sweat EtG as a function of consumed ethanol. First and foremost, this device will provide

researchers with a tool to better evaluate that relationship in more detail, with the hope being that a device is realized that provides a more complete and quantitative indicator for ethanol expression and its potentially harmful metabolites to allow for improved decision guidance in prospective users.

The proposed device would function in the following way: The user would place the device on themselves with a fresh test strip. The user's pre-drinking baseline would be stored to calibrate the drinking session to their current sweat physiology. Based on the user's physiological metrics, and desired application of the technology a predetermined "safe" EtG threshold(s) will be set to report to the user once that threshold has been reached. After the consumption of alcohol, the sensor will not immediately respond, as ethanol is expressed first. As ethanol starts to metabolize in the liver, EtG will gradually rise in expression before peaking after the peak of ethanol. The device will identify this increase in expressed EtG by evaluating the rate of change in impedance response to estimate the relative concentration of EtG in the sweat. When EtG begins to reduce in expression the rate of change will begin to reduce in magnitude as observed in Figure 3D. The device will be programmed to determine once an acceptable level of EtG has been reached through the conversion of the rate of impedance change to EtG present in the user's sweat. It will take further investigation to determine these levels. To improve the accuracy of the device, it can be implemented in conjunction with current TAC monitors to provide a reference ethanol level for more accurate EtG calculations, as opposed to making it purely an alternative to TAC sensors. Figure 4 highlights this proposed device functionality. The flow chart highlights the device-user interaction and how it will report to the user when they are safely able to continue their daily regime.

Conclusion

Here we demonstrate for the first time a wearable sensor that can differentiate EtG concentrations in synthetic human sweat correlating to the predicted alcohol consumption profiles of moderate drinkers. The device performed comparably to conventional benchtop instruments. Three scenarios were tested for continuous monitoring of EtG, estimating the consumption of 1, 2, or 3 simulated drinks in a single hour. The device was not only able to detect and respond to EtG present in the samples, but also able to differentiate each test scenario from one another. It shows the ability for this device to act as an accurate reporter of EtG levels in low volume sweat, and its potential use to understand with more granularity the disposition of ethanol and its metabolites after alcohol consumption compared to standard BrAC and TAC measurements. This device could be deployed for use in populations at health risk with frequent moderate alcohol consumption. However, in the short-term the device provides a means for researchers to better understand the relationship between sweat EtG levels and consumed alcohol, which could serve as a much more robust indicator for the long-term impact of alcohol consumption compared to ethanol measurements alone. With further testing of this technology, it is feasible that this EtG assay could be implemented with other non-

invasive samples such as urine, as our group has demonstrated previously for similar biomarkers (Kamakoti, Kinnamon, Choi, Jagannath, & Prasad, 2018).

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Conflict of Interest

Drs. Shalini Prasad and Sriram Muthukumar have a significant interest in Enlisen LLC, a company that may have a commercial interest in the results of this research and technology. The potential individual conflict of interest has been reviewed and managed by The University of Texas at Dallas, and played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report, or in the decision to submit the report for publication.

Table 1. Drink scenarios and associated dosing concentrations of EtG

# Drink / hour	Applied EtG / 5 min (ng/mL)		
	0-25 min	25-85 min	85-110 min
1 drink	0	13.3	0
2 drinks	0	26.7	0
3 drinks	0	40	0

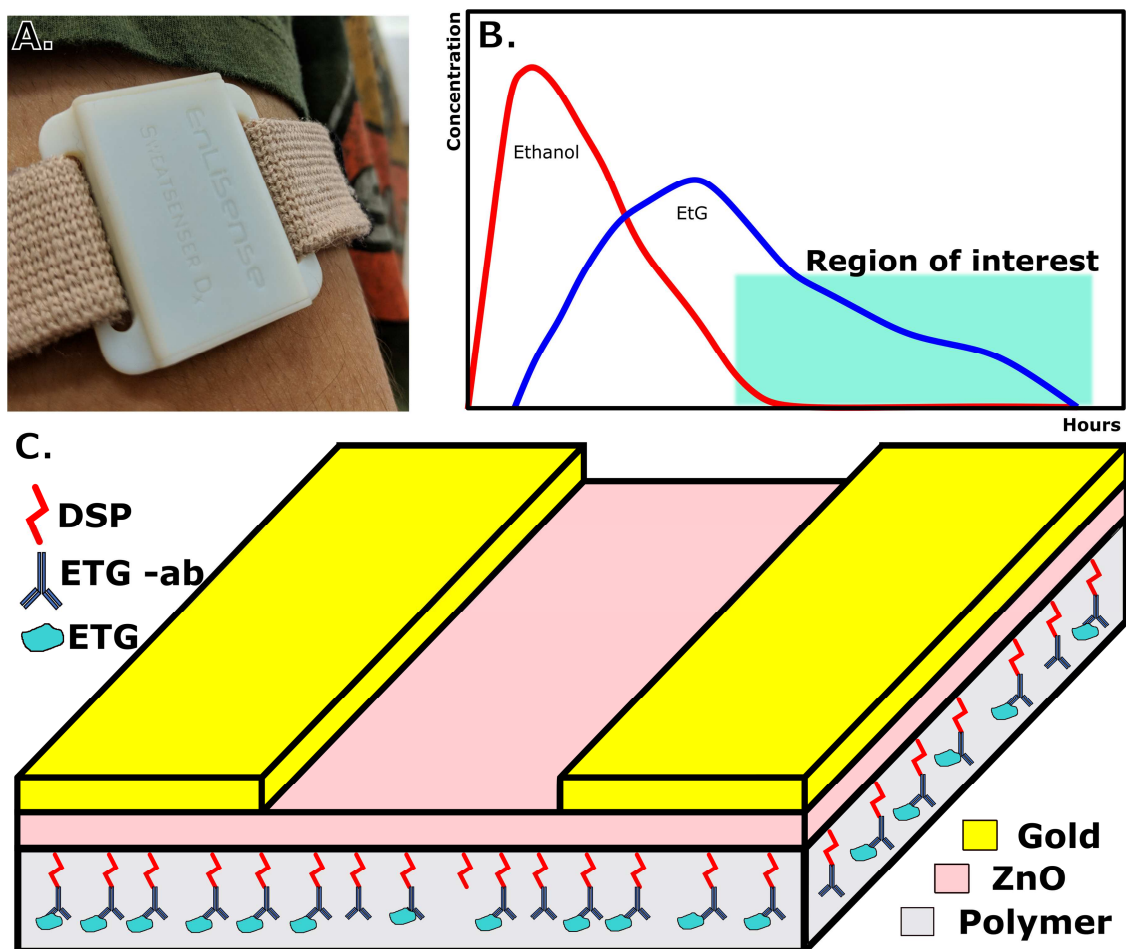


Figure 1 A) Picture of AWARE device mounted on a test subject to show form-factor and wearability of the device. B) Graph showing typical time course expression of ethanol and EtG in blood. Adapted from real data (Hoiseth et al., 2007) C) Schematic of the EtG affinity assay constructed on the porous polymer strip for disposable testing of EtG in sweat.

Benchtop v. Wearable

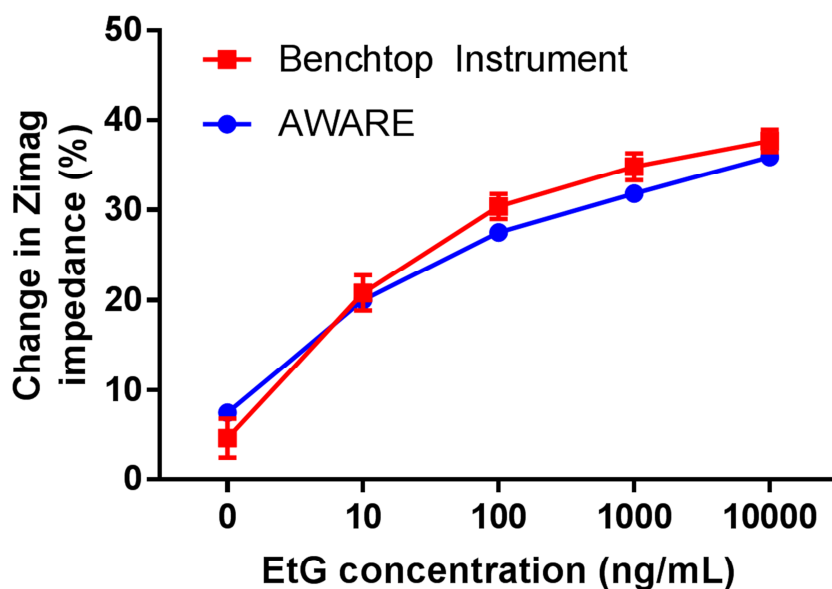


Figure 2 Calibration dose response of EtG comparing the benchtop potentiostat and the wearable AWARE platform. Tested concentrations of EtG (0, 10, 100, 1000, 10000 ng/mL of EtG). Error bars are standard error of mean. Error bars not visible on all data points.

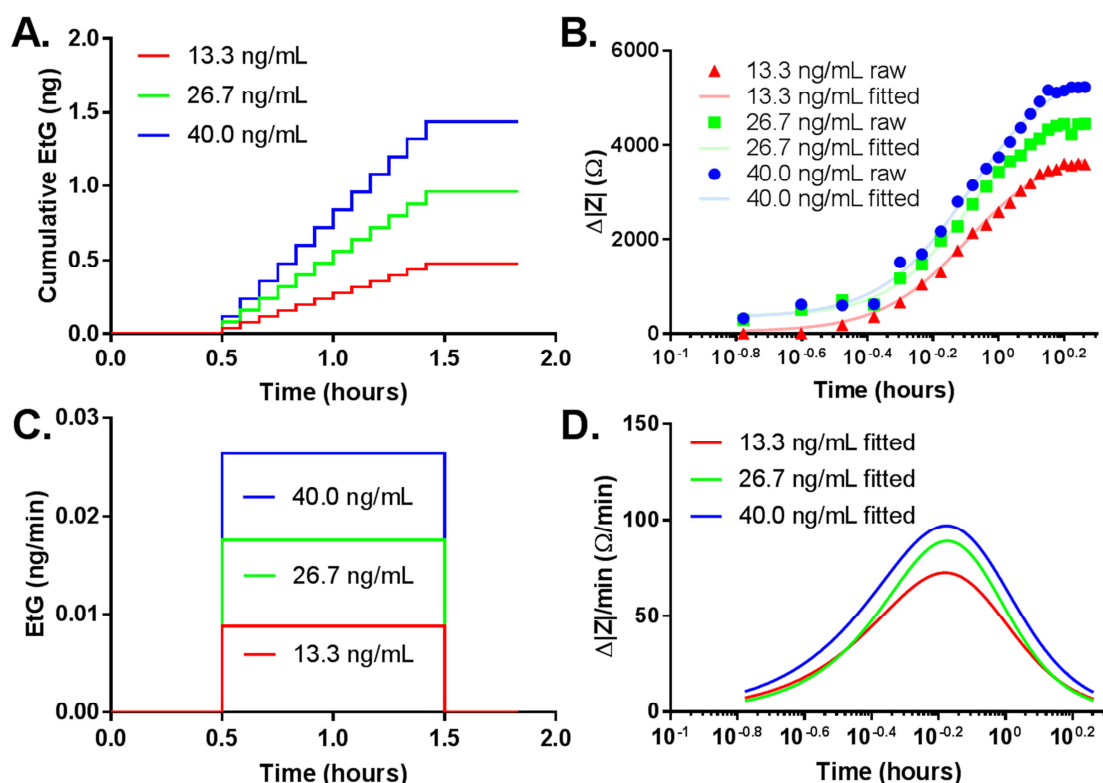


Figure 3 **A)** Cumulative addition of EtG to the sensor by weight for each of the three test scenarios over the time course of the experimental protocol. **B)** Change in impedance response for the three test scenarios on the AWARE device. The raw data (points) was fitted with a logarithmic function (line). **C)** Addition of EtG to the sensor per unit time for the three test scenarios. The rate of change in impedance from A. **D)** Change in impedance per unit time for the three test scenarios. The rate of change of the fitted lines in B.

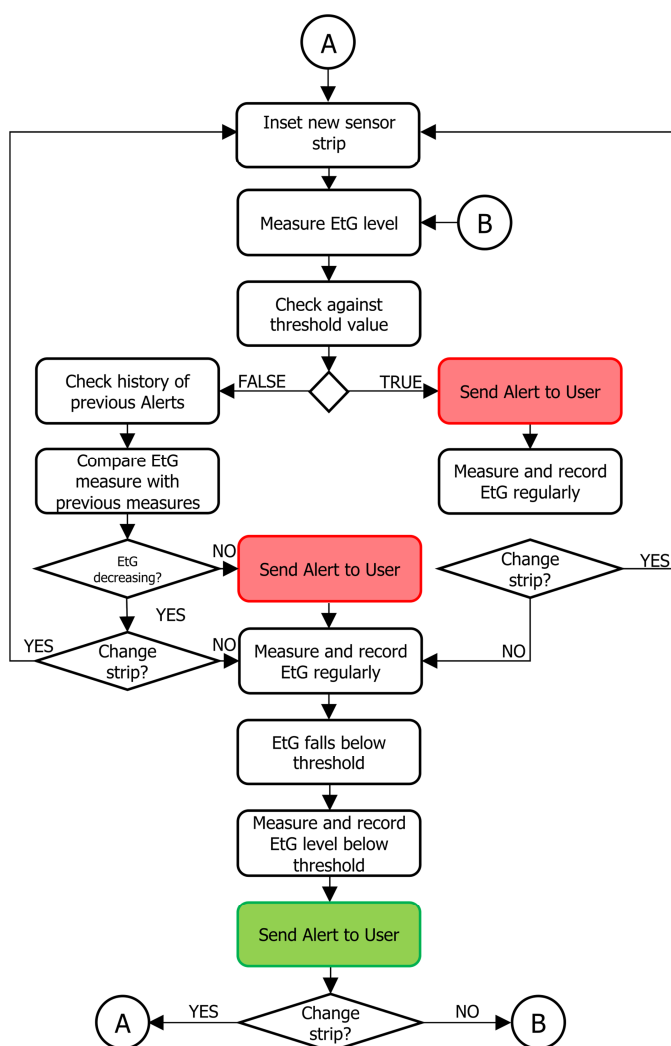


Figure 4 Flowchart detailing the proposed functionality of the AWARE device, and how it will decide when to alert the user of safe EtG metabolism levels to continue daily regime.

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