Reusable electrochemical impedance spectroscopy biosensor for the detection of cortisol in sweat: Introducing novel techniques suitable for future affective wearable devices and emotional stress.

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Declaration

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification, and that it is the result of my own independent work.

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Parts of this thesis have been previously published in Zamkah *et al.* (2020)

Abstract

Skin conductivity is used in emotion and stress-detecting systems based on physiology (sweat). However, these technologies do not detect sweat biomarkers or utilize sweat's biological information. Stress-induced volatile organic compounds (VOCs) cannot be detected using these methods. This study explores biomarkers of human emotional stress and identifies key indicators for wearable sensors in affective systems. Crime, health, the economy, and quality of life are all affected by emotional stress. Blood cortisol testing, electroencephalography, and physiological parameter techniques are the gold standards for stress measurement; nevertheless, they are expensive, inconvenient, and impractical for wearable real-time stress monitoring, such as a smartwatch, due to their single-use design. Instead, sweat cortisol was found as the critical stress biomarker for wearable affective system sensors in this study. Modern sensor research aims to create synthetic receptors with similar selectivity and sensitivity to natural antibody-antigen behaviour. This molecular recognition could lead to selective, sensitive sensors that can identify and monitor targets noninvasively when paired with modern methods for monitoring recognition element modifications. Molecularly imprinted polymers, MIPs, are synthetic antibody-antigen systems. They selectively bind their production molecule using a "lock and key" method. MIPs may offer biological receptor specificity and selectivity with environmental durability and low cost.

The current study explores the feasibility of using MIPs technology to detect cortisol in sweat for real-time monitoring of emotional stress episodes. A conceptual approach is given to make MIPs sensors more usable for monitoring cortisol sweat in wearable devices. As seen in the reviewed literature, cortisol and MIPs are under-researched biomarkers and their biosensors from the reviewed literature. Experiments employing electrochemical impedance spectroscopy techniques on a capacitance MIP confirmed this theory. It successfully detects cortisol within the physiological range as the higher response is recorded for a greater concentration. The literature also shows that no MIP biosensor is reusable in portable electronics. This work used a function generator simulation to evaluate the hypothesis that the target extraction technique employed

during the MIPS fabrication step is repeatable and suitable for employment in wearables.

Publications associated with this research.

Zamkah, A., Hui, T., Andrews, S., Dey, N., Shi, F. and Sherratt, R.S., 2020. Identification of suitable biomarkers for stress and emotion detection for future personal affective wearable sensors. *Biosensors*, *10*(4), p.40, 10.3390/bios10040040

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Chapter 1: Introduction

Consider John, a little boy of four years who has autism spectrum disorder (ASD). John's dad, Alfred, has turned on the living room light because he cannot find his keys. John, however, has photophobia whenever there is much light around. John's meltdown and aggressive behaviour, which resulted in a hand injury, occurred because he could not communicate his situation to his parents due to his disability. John's doctor believes that his anxiety and meltdowns are an automatic response to the sensory overload caused by a rise in cortisol. Therefore, John's doctor recommended reducing his exposure to light and keeping an eye on his stress levels. On the way home, Alfred stopped at a medical supply store to pick up a cortisol-monitoring wristband.

One night during the hot summer, Alfred forgot to draw the curtains, and when morning came, John was blinded by the sun's rays. His wristband began to alarm and transmit notifications to Alfred's phone, letting him know that John was becoming stressed and may have been on the edge of a meltdown. In a snap, John drew the curtain, giving him a relaxing fidget to calm down. This band acts as a secondary monitoring system for Alfred's son, notifying him of any signs of distress and allowing him to act before a complete meltdown occurs.

1.1 Background and aim

The originator of stress research, Hans Selye, defined stress as *"A non-specific response of the body to a demand"* in his book *"The stress of life"* (Selye, 1956). Selye's definition was incredibly general. Consequently, stress has numerous contextual definitions. It has been a hot topic in numerous medical, social, and psychological studies (Mawardi, 1979, Kessler, Price and Wortman, 1985). In Behavioural science, stress *is the discomfort induced by recognizing a threat* (Fink, 2017). Stress is the stimulus that induces the production of cortisol and adrenaline in neuroendocrinology (Miller and O'Callaghan, 2002).

Homo sapiens required these systems to survive life-threatening conditions such as natural disasters and animal attacks from the beginning of human history. The stress reaction is our survival mechanism, often known as the fight-or-flight response. Sappho, one of the finest ancient Greek poets, detailed this response for the first time

in human history, between 700 and 600 B.C., in her renowned song describing her emotional response to the prospect of seeing her beloved (Papi *et al.*, 2021).

"That man seems to me to be equal to the gods
who sits opposite you
and listens nearby to your
sweet voice and
lovely laughter, which indeed
sets my heart fluttering in my breast;
for when I look at you even for a moment,
then it is no longer possible for me to speak
but my tongue is broken
and at once a subtle fire runs beneath my skin
I cannot see anything with my eyes,
and my ears hum
a cold sweat comes over me, a trembling
seizes me all over, I am paler
than hay, and it seems to me
that I am little short of dying
but everything can be endured, since.."

- "Ode to Jealousy" by Sappho, 700-600 BC

Despite the poem's poetic description and exaggerated emotional tone, we can deduce that its author was aware of the physical manifestations of the fight-or-flight reaction, including rapid heart rate (HR), blurred vision, and emotional sweating (Papi *et al.*, 2021).

In the modern era, with the increase in stressful events, stress has become a defensive survival shield and a double-edged sword that might kill us (Daniel, 2020). To explain this paradox, during stress reaction, the human bodies produce several hormones, including adrenaline and cortisol, as a defensive reaction (Vining *et al.*, 1983). But, if the reactions are unnecessary, insufficient, or faulty, they may be harmful to the body or may cause chronic medical conditions (Selye, 1980), such as anxiety, depression (Hammen *et al.*, 2009), diabetes and obesity (Naik *et al.*, 2021).

Cortisol, commonly known as the stress hormone, is a glucocorticoid steroid hormone produced in the hypothalamus in the brain and the pituitary gland by the adrenal cortex to the blood and circulated to the body's organs and tissues (Daniel, 2020). Throughout the day, cortisol levels will naturally rise and fall in sync with the circadian rhythm, maintaining the equilibrium necessary for proper bodily function. During the morning hours, concentrations peak, and they gradually decline as the day progresses and night falls (Walsh and Dayan, 2000).

Cortisol plays a key role in a range of body functions, including metabolism, the glucose level in the blood and memory. Increasing the cortisol level in the body leads to several health problems, including fatigue, concentrating difficulties (Fraser *et al.*, 1999), and diabetes (Naik *et al.*, 2021), as well as a reduction of the quality of life (Alcalar *et al.*, 2013). Also, abnormal cortisol production in the body leads to Cushing syndrome (Alcalar *et al.*, 2013). Stress, adrenal cortex failure, psychological problems, and diseases like cancer all lead to increased production and release of cortisol (Rice *et al.*, 2019). Disruptions in the physiological processes governed by cortisol, such as metabolic functions and cardiovascular disease, have been related to abnormalities in its levels (Hogenelst, Soeter and Kallen, 2019), which can lead to heart attacks by increasing heart rate and hypertension (Sekar *et al.*, 2020), and anxiety and depression (Zea *et al.*, 2020). At exceptionally high rates, cortisol can cause disorders such as Cushing's syndrome, which appears as abdominal and facial obesity, hypertension (Daniel, 2020), weak muscles, bone loss, and fractures (Sekar *et al.*, 2020). In contrast to high levels of cortisol, weight loss, low blood pressure, muscle weakness (Sekar *et al.*, 2020), vomiting, weariness (Zea *et al.*, 2020), and excessive pigmentation (Singh *et al.*, 2014) are all symptoms of Addison's disease, which is caused about by low levels.

Cortisol levels, whether excessively high or low, can be used as a window into underlying physiological problems (Singh *et al.*, 2014) and serve as early warning signs for diseases such as Cushing syndrome. The clinical practice requires stress biomarkers to indicate cortisol availability and activity in the body for acute emergencies or for treating chronic conditions. Although, unfortunately, these biomarkers are not yet accessible (Daniel, 2020), monitoring cortisol levels consistently and noninvasively should be highlighted as a measure of prevention, monitoring, and diagnosis.

This monitoring has shown to be quite helpful since it enables real-time results and eliminates the need for trained professionals and visits to healthcare facilities (Sekar *et al.*, 2020).

1.2 Motivation

Conventional wisdom holds that stress occurs when an individual goes from a relaxed to an aroused state, setting off a chain reaction of protective physiological responses. Once the danger has passed, the body's physiological state returns to normal (Bräuner *et al.*, 2019). However, psychological hazards are more common than physical ones in the modern world, making stress a constant companion. When our bodies go through the same stressful situations repeatedly, it can be detrimental to our health eventually. Stress can be mental, emotional, or bodily, making it difficult to quantify.

Chronic Stress, if left unchecked, may have damaging effects on one's health (Kumar *et al.*, 2019). Work-related stress has been linked to coronary heart disease and high blood pressure (Fraser *et al.*, 1999; Vogt, Hagemann and Kastner, 2006). Stress can suppress the immune system and cause several diseases (Kumar *et al.*, 2019).

In addition, Clinicians have noted for a long time that chronically traumatized individuals usually have alexithymia, which is difficulty identifying and categorizing their emotional states, as well as orally expressing their emotions to others (Alvarez *et al.*, 1991). In this regard, Studies show a positive correlation between alexithymia and stress disorders. People who are unaware of their feelings have a more challenging time calming down when stressed. Tension can arise among individuals when they see a potential threat in their relationships. Suppose they cannot determine the origin and character of this feeling. In that case, they lack the adaptive mechanisms necessary to deal with the possibility of experiencing negative emotions. For example, being aware that one is feeling humiliated as a result of the opinions of others enables that person to explain that he was hurt and encourages the other person to seek clarification or avoid making comments of a similar nature in the future. A conversation of this nature is impossible without the participant feeling humiliated. In such a circumstance, shame will be experienced as "tension," "pain," or, at best, as another feeling (such as rage), which will increase the possibility that aggression will be considered one of the few methods to protect oneself from the source of such suffering (Elison, Garofalo and

Velotti, 2014). Also, poor emotional awareness might cause self-harming behaviours (Linehan, 2012). While alexithymia is a symptom of difficulty controlling emotions, the problem goes far deeper. One of the most widely accepted conceptualizations of emotion dysregulation describes it as including the following symptoms: a lack of awareness for the understanding of emotions, rejection of emotional responses, difficulty with goal-directed behaviour when upset, incapacity to control negative emotions and/or access to ineffective coping mechanisms for dealing with them (Gratz and Roemer, 2004).

The relationship between stress and alexithymia has been studied widely but not on real-time monitoring levels. A study shows an association between alexithymia and the stress hormone cortisol level using a laboratory salivary test (de Timary *et al.*, 2008).

The past decade of research has used speech, facial expressions, body language, and physiological factors to deduce people's emotional states (Hui and Sherratt, 2018). Physiological signals have been proven to be more trustworthy in providing a genuine evaluation of the human emotional state since they are difficult to be influenced by humans. Stress induces a significant physiological response in the human body, making physiological signals particularly well-suited to monitoring this emotion (Zhao *et al.*, 2018). However, the ‘flat’ affect is characterized by a fixed facial expression, a lack of gestures, and emotional reactivity. These symptoms are associated with different mental health issues, injury, and depression (Malá, 2008). Subjects with a more frequent flat affect may no longer react to the cortisol response in stressful situations (Lawler *et al.*, 2019). The cortisol response helps to survive. Conversely, adapting lack of response causes the flat affect patients to lack danger awareness that might be deadly. Having a technology that can respond to the stress reaction could assist patients in improving their emotional awareness and communication with people around them.

People with autism spectrum disorder (ASD), for example, have difficulty recognizing the emotions of others as well as understanding and managing their own (First, 2013), which is critical to responsive communication and social connection. In addition, flattened affect is recognized as a primary characteristic of autism (Stagg *et al.*, 2014) . Therefore, the literature uses a variety of techniques, including computer-human interaction such as virtual reality (VR) (Farashi *et al.*, 2022), video games (Beaumont *et*

al., 2021), and interactive robots (Özcan *et al.*, 2016), to promote emotional awareness in people with ASD.

In addition, a study provides a wearable device that uses many biosensors, including cardiovascular, skin conductivity, and accelerometer, to predict violent behaviour in children with ASD. With an average detection rate of 84%, the study demonstrates a respectable level of accuracy. However, it proposed additional data collection to improve the device's precision (Goodwin *et al.*, 2019).

The abovementioned emotional communication problems inspire this thesis to repeatedly determine the optimal biomarker for measuring emotional Stress in real time for wearable devices. Then, examine the essential biological recognition technologies to detect the target biomarker. In addition, more than 50 million people around the world suffer from epilepsy (Epilepsy, 2022), making it one of the most common neurological conditions. About 1.16 out of every 1000 people with epilepsy will die from Sudden and Unexpected Death in Epilepsy (SUDEP) (CDC, 2022). Memory and sensory loss, anxiety, and depression are only some of the physical, psychological, cognitive, and social effects that can result from this illness (Cano-López and González-Bono, 2019). In addition, several studies show that cortisol levels in the blood, saliva, and sweat are elevated in response to stressful events and that cortisol has the potential to serve as a biomarker for the avoidance of SUDEP (Herane Vives *et al.*, 2015).

Patients with epilepsy have greater basal cortisol levels than healthy individuals, and reaction to chronic and acute stress increases cortisol secretions, thus, higher cortisol levels than normal, which may be the cause of seizures and, in some cases, SUDEP. However, there has been a lack of current research in this area, and some of the results produced are inconsistent among studies (Cano-López and González-Bono, 2019).

It is important to know that several factors regulate cortisol levels to gain more precise results while monitoring this hormone in individuals with epilepsy. A variety of factors, including the circadian cycle (Cano-López and González-Bono, 2019), lifestyle, diet, addictions, age, and gender (Alves and Santos, 2021) can cause abnormal cortisol secretions, skewing results and leading to misdiagnoses.

1.3 Objectives

The main objective of this research is to design a wearable molecularly imprinted polymer (MIP) that would detect emotional stress by detecting the stress hormone cortisol in sweat in real time. Also, provide a self-cleaning method for the sensor to provide longer usability. The specific objectives of this work are:

- Identifying the suitable sweat biomarkers for emotional stress detection in personal affective wearable sensors.
- Identify the appropriate sweat biosensor for detecting the suitable biomarker for emotional stress detection for personal affective wearable sensors and explain its properties.
- To create a cortisol-detecting MIP biosensor suitable for cortisol monitoring appropriate for the underside of a smartwatch.
- To create an innovative MIP biosensor cleaning process for the sensor that simulates the cycle voltammetric approach.
- Examine the electrochemical spectroscopy technique for personal affective wearable sensors.

1.4 Achievement

A literature review was conducted to answer the first question and found that the current biomarkers, including cortisol blood tests and other biofluid tests, are unsuitable for real-time monitoring and not reusable. The results are published as a literature review paper in the MDPI Biosensors journal (Zamkah *et al.*, 2020). In the published work, cortisol, and Volatile organic compounds (VOCs) have been identified as the most significant biomarkers ideal for monitoring emotional sweat using a wearable biosensor. However, due to the lack of affordable technology for VOCs biosensor, the pandemic circumstances, and the time needed to develop a body odor biosensor, VOCs were eliminated as a target of the study. As a result, we look at the electrochemical stress biomarkers available. We found that cortisol, which can be detected in sweat, is the most important biomarker. This research also looked at cortisol metabolites and other anti-stress compounds as potential additional sweat-based stress biomarkers. Methods for detecting cortisol have been developed and used in many relevant investigations.

However, the literature studies results have not shown any cleaning methods that might increase the usage of the wearable MIP. Hence the challenge of creating a washable MIP in a wearable device remains. Nevertheless, based on the technical method of the fabrication of the MIPs, researchers are using an electrochemical method called cyclic voltammetry (CV) to remove the cortisol from the manufacturing MIP. This extraction procedure was successfully simulated in the lab using a function generator and can be integrated into future wearable works, which answers the fourth question.

A novel molecularly imprinted polymer capacitive biosensor, capable of self-cleaning, was designed and sent for manufacturing for the electrochemical detection of sweat cortisol based on a review of the existing literature on the topic. Comprehensive test results are presented to demonstrate the effectiveness of the sensor. However, there may be a lag in the broad dissemination of sweat cortisol wearable devices because of the necessity for the technology to support the commercial viability of wearable devices that detect sweat biomarkers. Therefore, for proof of concept, we offered a detection technique predicated on enhancing the viability of capacitive detection on a wearable device.

1.5 Thesis structure

This thesis has seven primary chapters. The literature reviews the suitable biomarkers for emotional stress for wearable biosensors and the associated biosensors in Chapter 2. Chapter 3 discusses the MIPs' literature review and their principles. They were followed by the experimental details and techniques presented in chapter 4. Chapter 5 will present the findings of the experimental procedure. The results will be discussed in Chapter 6 following their presentation. Finally, in chapter 7, a summary of this work will serve as its conclusion.

Chapter 2: Literature review

Parts of this thesis have been previously published in Zamkah *et al.* (2020)

2.1 Introduction

For many years, scientists have known that emotions can be communicated among animals by changing their body odors (Todd, Atema and Bardach, 1967). In stressful events, such as being injured or in life-threatening situations, chemical bio signals are released from the skin to warn other animals to escape or to gather. For example, Valenta and Rigby (1968) showed that rats can differentiate between stressed and relaxed rats using airborne odor. Therefore, it has been postulated that such effects may be extended to humans. Many experiments have been conducted to determine the role of human odors in emotional communication. Consequently, it is now known that humans can smell several emotions, including happiness (Chen and Haviland-Jones, 2000), fear (Ackerl *et al.*, 2002), and anger (Novaco, 2016). Indeed, Benderly (1988) stated that *"olfaction is our most emotional sense".*

In addition to body odors, physiological changes such as heart rate, skin conductivity, and oxygen saturation in the human body occur as an emotional response. Hui and Sherratt (2018) used physiological sensor data to detect emotional events based on the concept of emotional context awareness. Happy and Routray (2015) used image processing to detect emotional states in facial expressions. Li et al. (2019) merged facial image processing with electroencephalography (EEG) for improved emotional state detection, indicating that affective systems benefit from being multimodal. Yang et al. (2020) demonstrated emotion detection through speech for AI-based home assistants. While there is a large research area around affective systems and their impact on emotion and stress, this paper will review the literature, specifically looking for identified biomarkers in sweat that could be used to improve future affective sensors' sensitivity or classification to detect stress and emotion.

2.2 Emotional stress

In the modern era, stress has been identified as a significant factor that affects health, the economy, and quality of life (Singh *et al.*, 2014; Hung and Picard, 2016; Cano-López and González-Bono, 2019). Researchers have recognized the relationship between the emotions of an individual and their health, which in turn has raised the

subject of recognizing emotional status through affective computing (Picard and Healey, 1997). These emotions were classified by some researchers into six basic emotions, namely fear, disgust, joy, anger, sadness, and surprise (Hung and Picard, 2016). Recently, stress has been added to the recognized emotion set, which can be defined as the feeling caused by emotional tension, which might happen in certain circumstances when one reacts to demand or pressure that does not match with knowledge and experience or is over their capability. In the modern world, stress is a crucial problem. For example, researchers have reported that a growing number of community violence cases are related to anger resulting from stressful experiences (Bergman, Christopher, and Bowen, 2016; Novaco, 2016; Strasshofer *et al.*, 2018). Furthermore, police officers who do not cope with stress and its consequences have been shown to have increased rates of post-traumatic stress disorder and increased aggression (Bergman, Christopher, and Bowen, 2016). Also, stress has been shown to harm human health and plays a key role in diseases related to a mental disorder, such as anxiety (Tsukuda *et al.*, 2019), and seizures (Patrick Mannion, 2016; Cano-López and González-Bono, 2019).

2.3 Stress detection

Because of these risky influencers of stress, researchers have focused on overcoming the issues and detecting stress as early as possible to prevent further development. Although the classic invasive blood cortisol tests are the gold standard for measuring stress that includes the commercial Enzyme-linked immunosorbent assay (ELISA) (Shimada *et al.*, 1995). In addition, there are two major methods that have been used to detect stress noninvasively, either measuring brain waves via implementing EEG electrodes or utilizing biomedical tools to detect physiological bio signals, such as heart rate (HR), blood pressure (BP), and body temperature, and by using sweat sensors to measure skin conductivity (SC) (Kumar *et al.*, 2019), and electrodermal activity (EDA), heart activity through electrocardiography (ECG), muscle activity using electromyography (EMG), and respiratory response using electromagnetic generation. Furthermore, other research has involved advanced physical measurement methods to detect stress, such as automated facial expression analysis (AFEA) to monitor stress facial expressions that might also include infrared (IR) for eye movement tracking. Also, some computerized gesture analyses (leveraging

AFEA) for body gestures have been evaluated for their ability to detect stress. Another method for detecting stress involves comparing stress indicators such as voice patterns under stress to those under normal settings (Simantiraki *et al.*, 2018). Analysing how individuals use their mouse and type can potentially reveal their emotional state (Carneiro et al., 2017; Lau, 2018). Also, people can escape from stress by using their mobile phones, as research found a favourable correlation between stress and using a mobile phone (Sano and Picard, 2013).

In terms of device wearability, although EEG provides accurate readings and valuable information about the brain's states, its main disadvantage is that EEG electrodes must be attached to the scalp. Which is reported to be inconvenient for users (Gu *et al.*, 2018).

2.4 Sweat Biomarkers

While SC sensors are common in emotion detection systems, they are mainly used for measuring skin conductivity rather than the electrochemical content of the sweat. Sweat's electrochemical contents, such as the stress hormone cortisol and skin gases are significantly under-researched. Maintaining a healthy core temperature through perspiration is fundamental, but this bodily fluid also reveals interesting health data. Perspiration composition in healthy and rheumatic people has been studied (Ray and Mcswiney, 1934). This interest in sweat contents can be traced back to the early 20th century, when Silvers, Forster, and Talbert, (1928) noted the existence of glucose in sweat. Since then, scientists have investigated several characteristics of sweat, and have looked into several components of sweat, including fatty acids (Peter *et al.*, 1970), Ions (Prompt, Quinton and Kleeman, 1978) and viruses (Wormser *et al.*, 1992). In the modern era, with the rapid development of technology, researchers have been using technology to digitalise sweat biomarkers. Adewole et al. (2016) developed a sweat biosensor device to detect tuberculosis biomarkers. Kilic et al. (2017) fabricated a smart e-patch to monitor drug intake in schizophrenia. Upasham et al. (2021) developed a sweat biosensor to track the endocrine-inflammation relationship and monitor long-term disease. In addition, several glucose sweat-based biosensors have been developed recently (Khor *et al.*, 2022). Sweat comprises physiological components that execute physiological activities or participate as the end

product of the metabolite process, in addition to odd components detected as biomarkers. Chloride and Sodium play a significant part in the electrolyte balance demonstrated in sweat concentrations ranging from 10-90mmol-1, and exceeding this concentration may suggest cystic fibrosis, which also can be altered and affected by the bicarbonate level (Baker, 2019). While urea's physiological role is related to maintaining skin hydration, high concentration can be observed as a kidney failure biomarker (Sonner et al., 2015). The liver also converts ammonia to urea, and high ammonia concentrations can be biomarkers of Hepatic disorder (Guinovart *et al.*, 2013). While the body continues to eliminate ethanol as part of the metabolic process, an increase in ethanol concentration is associated with alcohol intoxication. (Baker and Wolfe, 2020). Moreover, as lactate is essential in skin hydration, exceeding its physiological range might involve tissue perfusion and pressure ischemia (Derbyshire *et al.*, 2012). Thermal disorders can result from the fluctuating cytokines presented in the sweat as an inflammation marker (Aranyosi *et al.*, 2021). Potassium also regulates the electrolyte balance and moisture of the skin; potassium level in sweat signals potassium consumption for hypertension patients (Houston, 2011). Calcium, a typical ion in sweat, can be associated with hypocalcaemia (Ahmed *et al.*, 2016). Glucose was one of the oldest components discovered in sweat. It is a significant biomarker for diabetes (Moyer *et al.*, 2012). In addition, sweat glands release Uric Acid, a final metabolite of purines. Thus, some studies showed abnormal concentrations resulting from cardiovascular or kidney disease (Baker, 2019). Another common sweat component is Vitamin C, which is initially the main factor in preventing scurvy disease (Sorice *et al.*, 2014). Finally, Amino acids, presented naturally in sweat, have been involved in some lung cancer screening studies (Calderón-Santiago *et al.*, 2015).

Meanwhile, research results have confirmed that sweat components vary among diseases, indicating the need for a metabolic analysis for general disease diagnosis, screening and monitoring, or personalized biomarkers (Luque de Castro and Priego-Capote, 2018). In the laboratory, the Wescore Macroduct (MCS) is the most common device that combines sweat sampling and analysis. Also, other liquid analytical devices such as liquid chromatography (LC), capillary electrophoresis (CE), and mass spectrometer (MS). In addition, a gas chromatograph is commonly used to analyse the volatile organic compounds in sweat (Serag *et al.*, 2021).

This review considers the current state of the art in understanding biomarkers present in sweat under stress and emotional events. We present the most recent electrochemical sweat markers and skin VOC studies to hypothesize potential stress biomarkers for future affective technology sensors.

2.5 Physiology of stress

Perspiration's main role is to maintain the core temperature of the human body at optimum levels, which is important for survival, as increasing the core temperature to over 40 °C causes serious health issues and can lead to death. The main objective of sweating is the downregulation of the body's core temperature in high-temperature environments or under physiological stress. However, there are other major roles for sweating, including gustatory sweating and emotional sweating (Wilke *et al.*, 2007). Emotional sweating occurs as a physical reaction against emotive stimuli such as stress (Machado-Moreira and Taylor, 2012).

In an event of exposure to acute stress, the human body initiates several behavioural and physiological responses, known as the fight-or-flight response, which includes several connected activated mechanisms that enhance survival in events of danger and maintain homeostasis. The sympathetic nervous system reacts to acute stress by sending adrenaline and noradrenaline signals that cause multiple physiological changes, such as increases in heart rate, blood pressure, and breathing rate (Ulrich-Lai and Herman, 2009). In a slightly slower timeframe, the hypothalamic–pituitary–adrenocortical (HPA) axis is activated, resulting in a production of the stress hormone cortisol as a part of increasing the circulation of glucocorticoids (Ulrich-Lai and Herman, 2009), as it can be seen in Figure 1. Emotional sweat is produced on the entire surface of the skin, but it is concentrated on the palms, soles, and underarms. All of these responses are relative, and as such, the level of response is based on several factors, including the nature of the stressor and the stressed person (Machado-Moreira and Taylor, 2012). Sweat from the palms and soles is usually caused by emotive stimuli, not by environmental temperature (Kerassidis, 1994). In comparison to thermal sweating, which can be affected by ambient temperature, emotional sweat does not change in response to the surrounding environment's temperature. It increases dependently and decreases during mental repose and sleep (Wilke *et al.*, 2007).

On the other hand, similar to thermal sweating, sole and palm emotional sweating involves the eccrine glands (Kerassidis, 1994). However, there is a lack of information regarding the central pathway of the eccrine glands, although some evidence has shown that the cortex and amygdala are involved (Asahina *et al.*, 2003). Interestingly, emotional sweating of the axillary area does not occur before pubescence, suggesting apocrine and apoeccrine glands play key roles in axillary emotional sweating, as they are inactive before this stage (Lonsdale-Eccles, Leonard, and Lawrence, 2003). Apocrine glands are activated by adrenergic stimulation and strongly respond to emotion (Nakazato *et al.*, 2004). However, the function of the secretion in these glands is unclear yet, although there is evidence that apocrine odors have similar effects to pheromones (Ackerl et al., 2002).

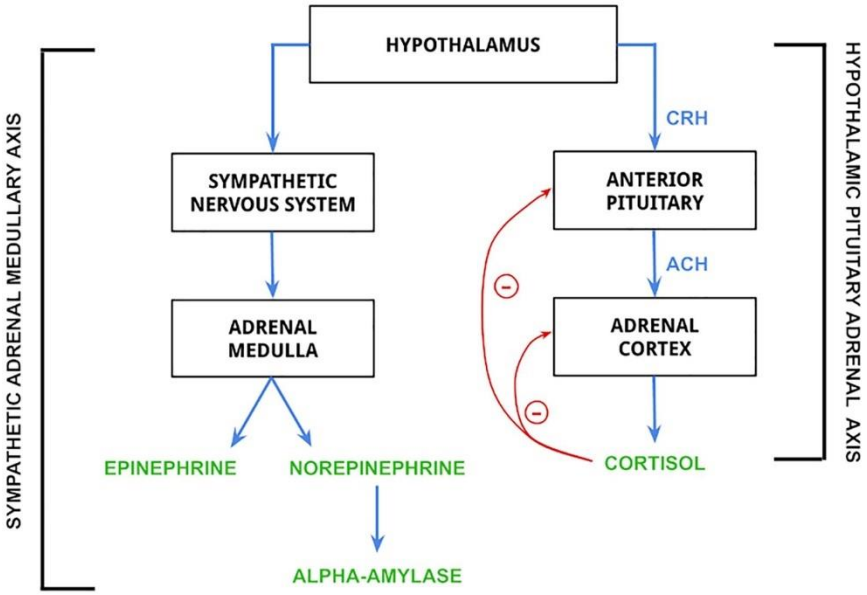


Figure 1 shows the michanism of fight-or flight respose, (Samson and Koh, 2020)

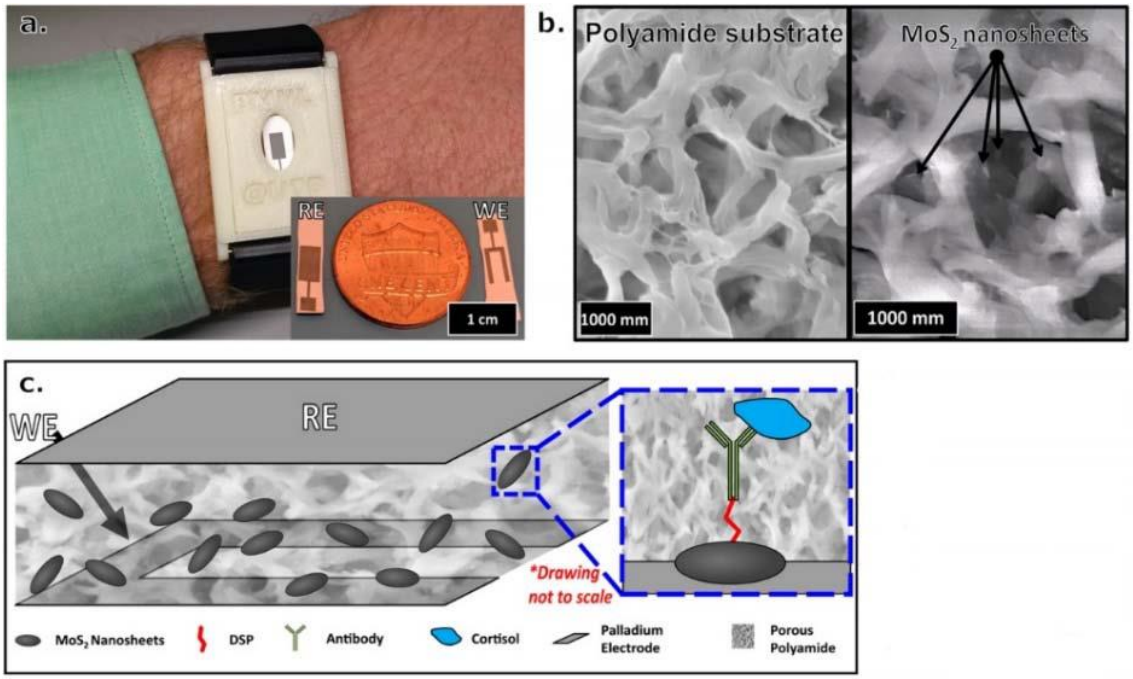


Figure 2 a) Visualized wristband device prototype for monitoring cortisol in human sweat. (b) Scanning electron microscope (SEM) image of blank polyamide membrane on the left side, where MoS2 nanosheets were placed into porous polyamide membrane on the right side. (c) Stack of MoS2 nanosheets within a polyamide membrane sensing platform for cortisol detection. The blue box is a magnified picture of a nanosheet that presents the affinity assay for cortisol, Kinnamon et al. (2017).

2.6 Electrochemical detection of stress

2.6.1 Antibodies

The slivery cortisol test has been identified as the most effective and promising non-invasive method to measure the cortisol level from biofluids (Singh *et al.*, 2014) in concentrations ranging between 8.16 to 141.7 ng/m (Jia *et al.*, 2016). Most recently, sweat has started to be an attractive area of research for measuring cortisol (Russell *et al.*, 2014). In 2016, researchers developed an antibody based wearable device using nanosheets of zinc oxide (ZnO) to detect cortisol in sweat at concentrations of 1 to 200 ng/mL. The study used a thiol-based linker molecule to bind to the ZnO (Munje et al. 2016). For low levels of cortisol volume detection, a portable cortisol sensor was developed using MoS2 sheets integrated into a nonporous flexible electrode system. The system succeeded in detecting volumes in the range of 1–5 µl. An immunity assay was designed, using MoS2 nanosheets operationalized with cortisol antibodies, seen in figure 2 (Kinnamon *et al.*, 2017). Most recently, CortiWatch, which is a wearable wristband with a watch shape, as can be seen in figure 3, was developed for monitoring cortisol fluctuations within the physiological range (8–141 ng/mL) for 9 h.

Although this device is a significant achievement in the field, it was designed to be disposed of after a low number of readings has been taken; this is interesting but not practical. The device has the potential to improve some medical applications, such as creating a circadian profile for a user and providing proof that self-monitoring of cortisol levels is possible (Rice *et al.*, 2019). Another recent study introduced an immunosensor that can detect cortisol and lactate using the label-free electrochemical chronoamperometric technique. This technique involves bioconjugation of cortisol and lactate antibodies with electro-reduced graphene oxide e-RGO, which is utilized as a synergetic platform for signal amplification. The prototype device can connect to smartphones via Bluetooth and can detect responses at concentrations as low as 0.1 ng/mL. In terms of selectivity, the device showed no cross-sensitivity between the two biomarkers or other components present in sweat (Tuteja, Ormsby and Neethirajan, 2018). Additionally, another cortisol detection immunosensor was introduced in a study using a miniaturized potentiate (M-P) chip (LMP91000) to perform a three-electrode range cyclic voltammetry (CV) measurement. The system succeeded in detecting cortisol in the physiological range, with a sensitivity level of 1.24 µM of cortisol (Cruz *et al.*, 2014). Additionally, a four-channel electrochemical impedance spectroscopy (EIS) analyser module was designed to detect cortisol in sweat. This module utilized flexible chemi-impedance sensors and was constructed with three gold electrodes for wearability. It was developed to detect cortisol in an ultra-low volume of sweat (1–3 µL) using an antibody-based technique, as well as to measure other physiological parameters, namely pH and skin temperature (Sankhala, Muthukumar and Prasad, 2018).

mHealth is another strategy in this subject. According to Torrente-Rodríguez et al. (2020), the wireless device measures cortisol levels in sweat using laser-induced graphene. It consists of three graphene working electrodes, an Ag/AgCl reference electrode, and a graphene counter electrode. Cortisol detection occurs when sweat cortisol binds to the graphene electrode, generating a cathodic current. To determine cortisol in sweat. The cathodic current on the electrode surface is measured to determine cortisol in sweat. Additionally, the study revealed critical investigations on the correlation between the perspiration and circulating cortisol for the first time. Also discovered was a clear correlation between cortisol levels in sweat, serum, and saliva.

Cheng et al. (2021) invented a flexible battery-free cortisol antibodies biosensor patch that detects sweat cortisol using near-field communication that enables the device to communicate with smartphones. For on-body testing samples, the equipment detected cortisol in real time and distinguished between emotional episodes and moments of relaxation. The device represents a promising advance in managing one's mental health. Naik et al. (2021) demonstrated a dual imprinted biosensor to detect cortisol only once and continue monitoring for glucose. Leitão et al. (2021) demonstrated an optical biosensor using surface plasmon resonance (SPR) technology to detect cortisol. The anti-body-based biosensor has shown a high selectivity to the cortisol as it can detect 0.005 ng/ml. Liu et al. (2021) developed a wearable nanostructured film biosensor for detecting cortisol in perspiration. Differential pulse voltammetry (DPV) is used to assess the detection's electrochemical performance. The detection limit demonstrates remarkable selectivity since it can detect as low as 1 fg/ml. Bianet et al. (2022) investigated the differences between the direct attachment of the cortisol antibodies to single-walled carbon nanotubes (sc-SWCVTs) and have gold nanoparticles as linkers between the antibodies and the nanotubes in a field effect transistor (FETs). The results showed that having a direct connection between the antibodies and the (sc-SWCVTs) improve performance. Demuru et al. (2022) developed a rapidly sensitive cortisol biosensor that can detect within 5 minutes. The antibody-coated transistor detected lower cortisol levels around 50 µA/dec in the 1–1000 nM.

Laochai et al. (2022) modified an electrochemical immunosensor electrode to facilitate cortisol antibody immobilization. The electrochemical signal of detection of this sensor is the reduction of the oxidation current using cyclic voltammetric CV techniques. The device can detect cortisol within the physiological range. Madhu et al. (2022) investigated a SnO2 nanoflake-integrated conductive carbon fibre (SnO2/CCY) to detect cortisol. Immobilized antibodies boost the system's selectivity. The CV technique was utilized to determine the extent to which electrochemical signals were reduced during binding processes. Finally, Santiago et al. (2022) built a three-electrode device for cortisol detection. Importantly, numerous layers of graphene oxides and cortisol antibodies were used to build the working electrode. Cortisol is also tested using the CV technique to identify the change in current.

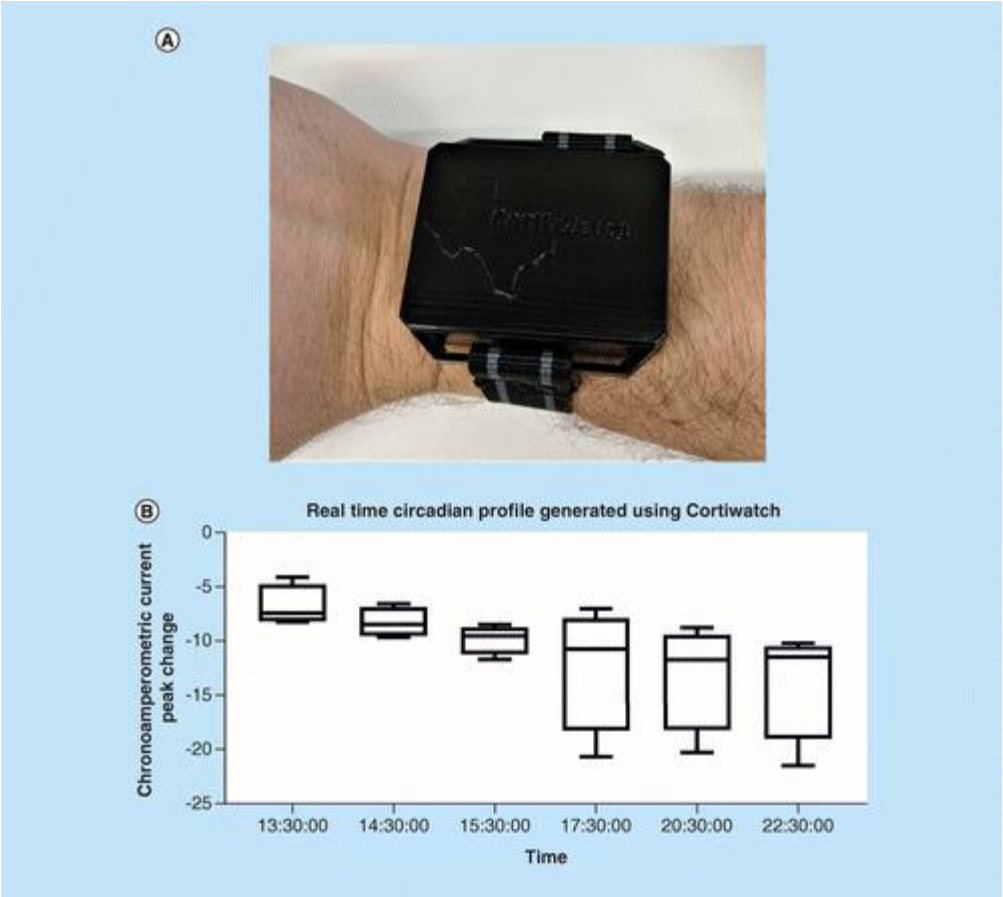


Figure 3

A) Wearable device prototype for CortiWatch B) it is used during experiments on human subject's Real-time rhythmic pattern obtained for a human participant throughout a 9-hour time (Rice *et al.*, 2019).

2.6.2 Aptamers

With the debut of SELEX technology in the 1990s, researchers were able to systematically evolve ligands by exponential enrichment (Moon *et al.*, 2015). The aptamer is a particular sequence of oligonucleotides isolated using SELEX technology from a library of randomly generated DNA or RNA (Mondal *et al.*, 2015). In addition to antibody-based methods, Aptamers biosensors are an alternative technique has been developed to detect cortisol in sweat.

This technique is a colorimetric detection method based on the conjugation of cortisol selective aptamers with the surfaces of gold nanoparticles (AuNPs). The aptamers react with cortisol molecules present in the sweat, provoking their desorption from the surface of the gold nanoparticles, resulting in reddish colour in AuNPs. The changes in

colour are due to the introduction of salt in the colloidal solution, which causes aggregation of AuNPs. The sensor detects within the physiological range of cortisol present in sweat, using the visual range of detection (1 ng/mL). There were no interactions with other biomarkers in sweat.

Wang et al. (2022) introduced a simple cortisol aptamer biosensor methodology. cortisol aptamers are bonded to AuNPS-DNA, when the cortisol is detected by cortisol aptamers it reduces the UV intensity absorption peak. The biosensor shows significant selectivity as it does not recognise any other tested samples. For more complex design, Weng et al. (2022) created a smartphone-based 3D microfluidic origami biosensor for the detection of cortisol in sweat. The biosensor consists of cortisol aptamers fluorescently labelled with carboxyfluorescein (AFM) and MoS2 nanosheets. The optical detector was able to detect a broad range of cortisol concentrations within the physiological range, as indicated by the rise in fluorescence intensity with increasing cortisol concentrations. Also, to monitor cortisol levels, An et al. (2022) created a disposable aptamer patch to monitor cortisol levels, as it can be seen in figure 4. Again, electrical measurement forms the foundation of the sensor concept. When cortisol binds to the aptamer, it shifts to the nanofibers' surface, altering the electrical characteristics, raising resistance, and boosting the NFs' conductivity. Thus, the labelled aptamer detection increases the intensity of the NFs.

Alternatively, Tu et al. (2020) provided colorimetric tests using blue tetrazolium to assess sweat cortisol in twenty human samples. The researchers discovered a substantial variation in cortisol concentrations between male and female samples, as well as between apocrine and eccrine cortisol levels. This distinction between glands can be important when selecting a wearable device for placement on the human body. Moreover, the aptamers technique has advantages over antibody-based methods in terms of stability, costs, and user-friendliness (Dalirirad and Steckl, 2019).

Figure 4 monitoring cortisol levels using a wearable aptamer-field-effect transistor sensing system (Wang et al., 2022).

2.6.3 MIPs

Parlak et al. (2018) presented a new patch-type multifunctional layered biosensor that was developed to detect cortisol in sweat. A molecularly imprinted polymer (MIP)-based artificial recognition membrane was developed to interpose between semiconductor polymer channels, typically, the poly(ethylenedioxythiophene)–poly (styrenesulfonate) (PEDOT–PSS) channel layer and sweat reservoir controls the selective transit of cortisol molecules from the skin to the organic electrochemical transistor (OECT) sensor channel. This molecule selective OECT (MSOECT) demonstrated physical and chemical stability at body temperature, as well as the capacity to resist physical deformation. The system was able to detect cortisol at low concentrations (0.1–1 µM) with no selectivity or specificity mistakes recorded.

Nonetheless, Mugo and Alberkant (2020) introduced a molecularly imprinted electrochemical (MIP) sensor to detect cortisol in sweat. The sensor was fabricated using layer-by-layer (LbL) assembly and based on flexible poly (glycidylmethacryate-co ethylene glycoldimethacrylate) (poly (GMA-co-EGDMA)). The MIP was built to suit the human skin as a wearable device, as well as to be selective for cortisol detection in human sweat. The sample was collected from the forehead of one volunteer after exercise. The experiment was repeated for both a MIP sensor and a nonimprinted polymer (NIP), namely a cortisol-free labelled film that was polymerized similarly to the MIP but without the addition of cortisol. The selectivity of the sensor has been shown to be blind to other interfering sweat components. In terms of selectivity, the MIP sensor succeeded in detecting cortisol effectively in human sweat in the range of 10–66 ng/mL. However, the sensor has a limitation in terms of detecting cortisol at a lower range (2.0 _ 0.4 ng/mL). In addition, a biosensor based on MIP has been

developed to detect cortisol in sweat with a fingertip touch. It is inspired by the large concentration of eccrine glands in the fingertips, which produce a substantial amount of perspiration that facilitates sampling. The sensing component is a screen-printed electrode with a MIP layer specific for cortisol. Due to Prussian blue redox probes that provide direct electrical signalling of the binding event, cortisol levels can be detected by amperometric detection. This study introduced a novel way to collect sweat using sweat-absorbing polyvinyl alcohol (PVA) hydrogel, unlike prior emotional sweat investigations that require a psychological or physiological stressor (Tang *et al.*, 2021). Mugo et al. (2022) reported an affordable dual biosensor to detect cortisol using MIPS technology and a PH sweat biosensor. The biosensor has detected 1.4 ± 0.3 ng/mL insensitivity. Also, the sensor's reusability is interesting, since it was reused 15 times every month with just minimal variations in response. After that, Mugo, Lu and Robertson (2022) adapted their technology to a more wearable form. The sensor is attached to cotton textile patch to be suitable for wearable design. The patched sensor was examined fifteen times over the course of a month and demonstrated satisfactory accuracy.

Yeasmin et al. (2022) introduced a gold nanoparticle-doped molecularly imprinted polymer for cortisol detection. The gold particles aim to boost the electron transfer between the redox probes and the electrode across the insulating MIP layer. Unlike capacitive MIPs biosensors of the former, this biosensor measures the change in current response triggered by the binding of target molecules to MIP, resulting in electron transfer. The gold nanoparticles play a crucial role in increasing the efficiency of the captured target to block the electron transfer. However, the biosensor is only tested to detect artificial saliva cortisol; further investigation to detect sweat cortisol is recommended to be suitable for wearable biosensors. Bian et al. (2022) recently introduced a disposal sweat cortisol MIP biosensor. The sensor was fabricated using a high throughput roll-to-roll R2R screen-printed electrode. The sensor was built as a batch for single use on human skin. The signal of cortisol detection was a reduction of the current signal compared with less concentrated samples. In terms of specificity, the developed sensor was made to detect cortisol from sweat produced by eccrine sweat, which contains various components. Therefore, minor structured or cortisol-similar components might bind to the MIP cavities. As a result of the selectivity tests, all tested components reacted less to the current versus cortisol. On the other hand,

the sensor showed a higher response to glucose than others, which might be because of a smaller structure of glucose, so they bind to the cortisol cavities. Also, the selectivity tests confirmed that the NIPs are not selective to cortisol. A new approach in the cortisol MIPs biosensor has been introduced recently by Yılmaz et al. (2022) where a plasmonic cortisol MIP biosensor is proposed. This integration between the technologies measures the change of the reflection light of the sensor angel during the measurement of different concentrations of cortisol the higher concentration of cortisol, the more significant rise in reflected lights. Even though the sensor effectively detected cortisol in other fluids, including artificial plasma and urine, it can perform only 5 times at full capability. Unlike the capacitive MIPs, Dykstra et al. (2022) and Duan et al. (2022) introduced differential pulse voltammetry (DPV) as a signal measurement method. When the MIP detects cortisol, the cavities are filled, blocking access to the redox probe to transfer to the electrode surface. This leads to reduced electron transfer, resulting in a reduced electrochemical signal. Thus, DVP is the quantification method of cortisol detection as the calibration curve correlation between the concentration of cortisol and its corresponding peak current change. So, the higher the slope of the calibration curve, the more sensitivity of the MIP to the target.

In comparison between the natural receptors and MIPs, the latter provides practical aspects over the natural recognition systems. On the other hand, MIPS seems more affordable, selective to the targets and provides environmental stability. Also, the natural receptors are disposed of while MIPs can be cleaned.

2.7 Non-Electrochemical detection of stress

Physiological stress signals are commonly used for stress detection. Most of their smartphone-compatible sensors can be recorded for visual and vocal analysis. Empatica E4 is a wristband medical-grade biosensor based on streaming and viewing real-time physiological data. It permits users to collect data on various physiological biomarkers, including EPA and heart rate variation HRV. Additionally, the device contains a three-axis accelerometer to detect motion (Indikawati and Winiarti, 2020).

Autosense is a pack of six sensors wearable devices in a portable form that allows for collecting cardiovascular and respiratory measurements via radio transmission and processing the data to detect stress stats (Ertin *et al.*, 2011). The Massachusetts Institute

of Technology developed the Q-sensor, a wearable device that detects stress by monitoring changes in skin conductivity. A *shimmer sensor* is a biosensor developed to measure stress in the workplace, which employs EDA sensors in the fingers and can transmit data to PCs through Bluetooth. *DataLOG* is a flexible EMG band worn on various body areas, making it applicable to a vast array of research (Pourmohammadi and Maleki, 2020). Even though prior devices were able to identify real-time measures of physical biomarkers, they are incapable of detecting emotional stress. The inability of these metrics to differentiate between stress and usual settings necessitates additional measurements to detect stress (Yoon, Sim and Cho, 2016). Therefore, recent studies and research demand stress biomarkers beyond the physical ones. They require a traceable physiological imprint of human stress (Zea *et al.*, 2020).

Hair cortisol has been used for measuring chronic stress as a non-invasive method (Cirimele *et al.*, 2000). Recently, Herane-Vives et al. (2020) used earwax samples to measure the cortisol level using a non-stressful, non-invasive, novel self-sampling method. The study found that the cortisol concentration in earwax is higher than in hair, and earwax has the potential to overcome some of the major limitations of the hair methods, such as stability and storing conditions, as earwax has bacteriostatic properties. However, earwax has been demonstrated to be a reliable source of cortisol, this method is not practicable for acute stress measures or daily monitoring wearable devices since earwax cortisol concentration is not impacted by acute stress (Bende, 1981) because of the nature of the ceruminous glands which is responsible for the secretion of the ear wax (Montagna, 1955).

When measured before and after periods of acute stress, leptin has been shown to be a reliable indicator of the body's response to stress (Bouillon-Minois *et al.*, 2021). Further research is needed to address leptin's behaviour after acute stress, as it was observed in another study that leptin increases significantly after acute stress (Tomiyama *et al.*, 2012). It is debatable whether leptin is an ideal biomarker for wearables, given that it has primarily found in serum and is made by fat cells. Finally, e-nose also has been involved in cortisol detection. A study used e-nose with an association of a pattern recognition software tool to detect a low concentration of cortisol (5 µM–50 µM) in sweat (García-cortés et al., 2014). In a previous work Zamkah et al., 2020 discussed this study saying "***here e-nose detected stress situations by measuring the concentrations of cortisol and adrenaline in sweat; however, no further studies have been carried out***

on this.". An e-nose utilizing metal oxide gas sensors, pattern recognition algorithms, and artificial intelligence technologies has been developed in response to this call (Durán Acevedo, Carrillo Gómez and Albarracín Rojas, 2021). Galvanic skin response analysis is connected to the system. The skin conductivity was measured at the fingertips, and the volatile organic compound emissions were recorded at the forehead. As can be seen from the data, there is a clear distinction between the stressed and relaxed samples. In addition, the system can distinguish between light, moderate, and severe stress. The integration of these technologies is an emerging method for investigating the e-noses to detect emotions, particularly stress.

2.8 Volatile organic compounds (VOCs)

Developing a non-invasive tool offering a significant level of selectivity and sensitivity with real-time operation is a challenging issue. For this reason, VOC sensing technology has been widely used in the medical field for several diseases that exhibit specific changes in the pattern of the VOCs of sweat (Hsieh and Yao, 2018). Various gases are released from human bodies, including metabolic gases, while sweat VOCs and VOCs are produced by floral bacteria (Sekine, Toyooka and Watts, 2007). Also, VOCs can be found in exhaled breath and other body biofluids (Drabińska *et al.*, 2021). VOCs have a high vapor pressure at ambient temperature and a low boiling point. Thus, they release many molecules into the air when they boil (Ciccioli, 1993). VOCs are generally nonreactive, and the adverse effects on human health depend on several circumstances, such as exposure duration and intensity. This involves continuous monitoring of an individual's VOC exposure. Accurate monitoring of VOC exposures could facilitate a deeper comprehension of the environmental and physiological conditions that govern human health (Mahmud *et al.*, 2021).

On the other hand, there is a lack of research on VOCs relating to human emotions, even though several studies have tested the role of sweat in human emotional interactions, such as fear sweat (de Groot, Smeets and Semin, 2015) and anger aggression (Novaco, 2016). These studies present the olfactory roles in emotional interactions, while the roles of chemical contents of emotional sweat had not been the focus of prior studies. Therefore, Zamkah et al. (2020) combined two major studies investigating VOCs stress biomarkers. One study hypothesized that stress biomarkers are released from the skin

in response to stress. The study used the trier social stress test (TSST) to measure stressors, a cortisol salivary concentration test as the gold standard for the study, and a survey as the result comparison tools. The gas analysis was performed by gas chromatography–mass spectrometry (GC/MS) system. The participants of this study were 30 females, as they are responding to TSST better than males. These subjects had a general anxiety tendency, which was evaluated using a physiological questionnaire. The subjects ranged between normal and high anxiety trait levels, reflecting the type of people who are likely to suffer from mental disorders as a result of stress. The study identified 6 stress biomarkers (1,2-ethanediol acetophenone, heptadecane, hexanedioic acid, dimethyl ester, benzyl alcohol, and benzothiazole) that were released from underarms of the samples (Tsukuda *et al.*, 2019). Table 1 depicts the released amounts of the six stress VOCs that were identified as stress biomarkers in the skin. In the same vein, another study used a deterrent methodology to identify stress VOCs. The paced auditory serial addition test (PASAT) was used as a stressor and sweat samples were collected from foreheads of 20 volunteers. The subjects were 10 males and 10 females between 19 and 26 years old. The samples were randomly separated into two sampling sessions. In the first session, subjects sat and listened to classical music. In the second session, subjects undertook the PASAT test. In addition, heart rate and blood pressure measurements were recorded. It was found that four stress biomarkers (benzoic acid, n-decanoic acid, a xylene isomer, and 3-carene) (Martin *et al.*, 2016). Notably, the identified biomarkers from both studies were different. However, in terms of wearability, there are no commercial biosensors available to detect stress via VOCs, but a study did recommend a nanomaterial-based sensor array for future wearable biosensors for VOCs, Figure 5 (Broza, Zuri and Haick, 2014). Unlike GC/MS, which identifies specific VOCs, this array relied on the collective pattern of VOCs. Gioia et al. (2022) also attempted to respond to our inquiry by conducting a preliminary investigation on the association between stress response and perspiration. The purpose of the experiment was to collect samples from the participants' underarms. Complex laboratory instruments have been utilized to analyse chemical data, and they were able to detect a wide range of volatile organic compounds. However, they chose to focus on acids because they had previously been identified by two prior research. Therefore, the study combines both chemical and physiological data (thermal imaging, electrocardiogram, and electrodermal activity). The data indicate that dodecanoic acid

is a possible biomarker for stress. In addition, additional locations and biofluids are recommended for future direction investigations.

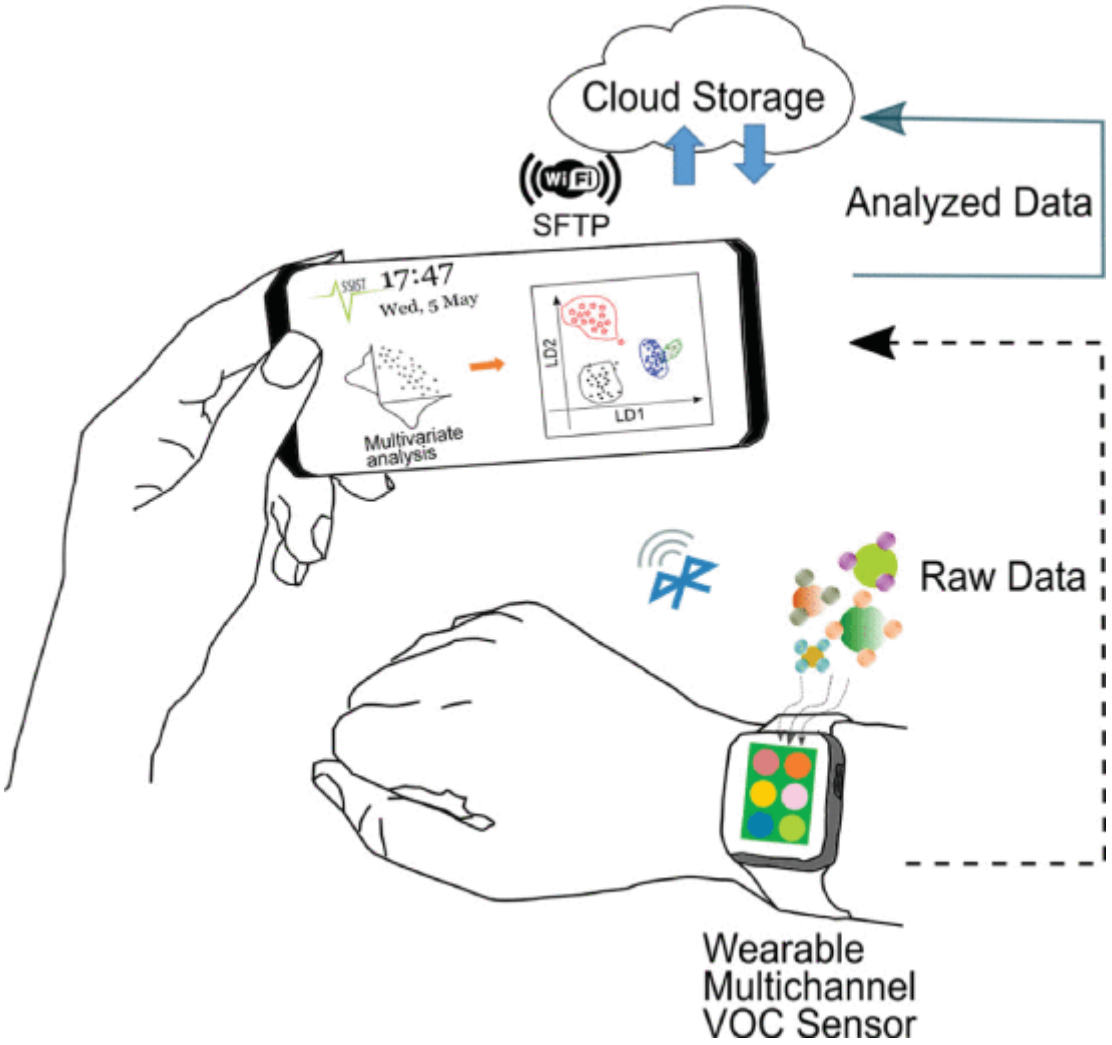


Figure 5 Multi-channel VOC sensor application conceptual diagram *(Mahmud* et al*., 2021).*

2.9 Results

The results show a notable development in the field of electrochemical stress bio signals from sweat. Several methods have been utilized to detect cortisol, the main stress biomarker. In this regard, the antibody-based technique is the most common tool used to detect cortisol in sweat (Cruz et al. 2014; Munje, Muthukumar and Prasad, 2016; Tuteja, Ormsby and Neethirajan, 2018; Ganguly et al., 2019; Rice et al., 2019; Cheng et al., 2021b; Leitão et al., 2021; Liu et al., 2021; Bian, Shao, Liu, Zeng, et al., 2022; Demuru et al., 2022; Laochai et al., 2022; Madhu et al., 2022; Santiago et al., 2022) , while less commonly used techniques include the aptamer (Dalirirad and Steckl, 2019; Martin et al., 2014; Pusomjit et al., 2021; Wang et al., 2022; Wu et al., 2022), e-nose (García-cortés et al., 2009; Cho et al., 2018), and MIP techniques which are significantly raised after the publication of the literature review in 2020 (Parlak et al., 2018; Mugo and Alberkant,

2020; Daniels et al., 2021; Villa et al., 2021; Bian, Shao, Liu, -, et al., 2022; Duan et al., 2022; Dykstra et al., 2022; Yeasmin et al., 2022). In terms of sensitivity, all the above-mentioned studies succeeded in detecting cortisol in its targeted range. However, different detection ranges were presented in the studies. The lowest levels of cortisol concentration detected were in 1fg/ml (Liu *et al.*, 2021) while the only manufactured antibody-based biosensor, CortiWatch, achieved a more modest level of detection, ranging from 1 to 150 ng/mL (Rice et al., 2019), and the only manufactured aptamer-based biosensor does not specify the detection level. Instead, the used a dynamic range (1pM to 1 µM) as a critical parameter.

From the perspective of the placement of wearable sensor devices, it is an advantage that the eccrine glands are spread over the whole human body, as this offers a variety of placement options. In terms of selectivity, no reported errors were mentioned in cortisol biosensor studies.

Cortisol metabolites have the potential to be sensed as stress biomarkers in wearable devices. The current methods to detect them require sophisticated lab-based machines (Runyon *et al.*, 2019). Further investigations are needed to create long-lasting sensors for wearable devices. Antistress hormones as stress biomarkers are also under-researched. However, a Zn+ ion biosensor has been developed to detect an antistress hormone called oxytocin in biological fluids for medical purposes (Mervinetsky *et al.*, 2019). From considering the literature, it is possible to recommend that more trial studies be conducted to detect the ranges and concentrations of biomarkers in sweat during a range of common stressful events, in order to further facilitate the capture of information needed in the design of biomarker sensors in future effective systems.

VOC technology is in the development stage. Two studies utilized different methodologies and found different stress biomarkers. The first study found changes in the concentrations of the biomarkers 2-hydroxy-1-phenylethanone, benzaldehyde, and 2-ethylhexan-1-ol in response to the stressor (Martin *et al.*, 2016), while the second study found changes in the concentrations 1,2-ethanediol acetophenone, heptadecane, hexanedioic acid, dimethyl ester, benzyl alcohol, and benzothiazole (Tsukuda *et al.*, 2019). However, in the first study, the biomarkers were found in the forehead samples, which might be produced from eccrine glands as cortisol metabolite VOCs, whereas in the second study the biomarkers were measured from apocrine glands (underarms). This difference might be the reason for the discrepancy between the experimental

results obtained. Another possible reason is that the VOC biomarkers resulted from floral bacteria (Tsukuda *et al.*, 2019). Further investigations are needed to understand the source of axillary VOCs, to also test the accuracy for both types of glands, and to test the performance of eccrine gland biomarkers at different places on the body."

Table 1 Summary of stress biomarkers from the sweat or skin, methods used to measure them, places

Biomarkers	**Methods**	**Place**	**Wearable Available**	**Potential Device**
Cortisol	Antibodies, aptamers, MIPs, and e-nose	Eccrine glands (Antibodies, aptamers and MIPs) Apocrine (e-nose)	Wrist band + patch	e-nose + Flexible
Cortisol metabolites	Labs	Eccrine glands	No	Flexible
Antistress hormones	Zn+ ions	Eccrine glands	No	Flexible
VOCs (Benzoic acid, n-decanoic acid, a xylene isomer, and 3-carene)(Martin *et al.*, 2016) 1,2-Ethanediol Acetophenone Heptadecane Hexanedioic acid, dimethyl ester Benzyl alcohol Benzothiazole(Tsukuda *et al.*, 2019)	Lab (GC/MS)	Eccrine glands. Apocrine glands or skin	No	Flexible

Various signals have been identified as stress biomarkers. Table 2 summarises the results that were found in this research. Cortisol has been the most popular stress biomarker in sweat, with eight studies having targeted cortisol in the physiological range of sweat. Three techniques have been utilized to detect cortisol in experiments involving antibodies, aptamers, or e-nose technology. Antibody recognition methods including immunoassay and electrochemical immune sensing were utilized in five out of eight studies to detect cortisol (Cruz *et al.*, 2014; Munje, Muthukumar and Prasad, 2016; Tuteja, Ormsby and Neethirajan, 2018; Ganguly *et al.*, 2019; Rice *et al.*, 2019),. These

methods were effective in terms of specificity to cortisol molecules because of the nature of antibody–antigen immunochemistry (Ganguly *et al.*, 2019). CortiWatch (Rice *et al.*, 2019), a cortisol wristband sensor, presents the antibody technique as an advanced step in this field. On the other hand, Wang et al. (2022) represent the aptamer technique as a developing step in the field. While many published works show patches and stickers suit the wearable devices as promising attempts (Yoon, Sim, and Cho, 2016; Sankhala, Muthukumar and Prasad, 2018; Cheng *et al.*, 2021b; An *et al.*, 2022; Demuru *et al.*, 2022; Mugo, Lu and Robertson, 2022).

In terms of placement, the antibody-based methods detect cortisol from eccrine sweat, creating a promising future for cortisol detection technology, as eccrine glands are present on the whole surface of the body, which ensures the flexibility of manufacturing pervasive wearable devices. Alternatively, aptamer methods provide a visual, rapid detection method to detect cortisol in sweat (Dalirirad and Steckl, 2019). The cortisol samples were, however, manufactured (i.e., no human body sample location was provided). Additionally, testing stress in real time has not been approved and finding suitable body placement locations for wearables have not been tested. Another cortisol detection method is e-nose, which “smells” the cortisol concentration in sweat vapours and uses additional pattern recognition tools to differentiate between stress events and quiet periods. Unlike the previous studies, sweat samples for this study were taken from the underarms of the samples, which means they were collected from apocrine glands. The sensitivity of the gas arrays increased directly with increasing cortisol concentration. However, a simplified wearable form of e-nose to detect cortisol concentration is not available. Samples were collected from apocrine glands (underarms), which could minimize the placements options, as apocrine glands are located in certain areas of the body, suggesting the potential for the development of wearable e-nose technology in “smart shirts” or armbands. Further studies are required to test e-nose technology for cortisol detection in eccrine glands, as succeeding in this would provide more fixable wearable options.

The combined response to stress of cortisol, its metabolites, and cortisone raises the idea of using multiparameter rather than only using cortisol, as all these markers are present in sweat within a measurable range. By using GC/MS techniques, all the markers can be separated from each other, and also, from other components of sweat, then

variable concentrations and patterns can be measured in stressful events (Runyon et al., 2019; Mugo and Alberkant, 2020) The samples in these studies were collected from eccrine glands, which indicates flexibility in terms of wearable device developments in the future.

Table 2 Comparison between three cortisol detection techniques over several factors.

Factors/Techniques	**Antibodies**	**Aptamers**	**MIP**
Selectivity	High selectivity to cortisol—no errors have been reported	High selectivity to cortisol—no errors have been reported	High selectivity to cortisol—no errors have been reported
Sensitivity	In the physiological range	In the physiological range	In the physiological range
Thermal stability	The lowest	High	The highest
Immune response	Can be rejected by the immune system	Cannot be rejected	Cannot be rejected
Cost	Expensive	Less expensive	The cheapest

2.10 Discussion

This chapter has highlighted previous work, showing that the detection of sweat cortisol and VOCs emitted from the skin are effective methods for detecting stressful events, and have huge potential to supplement emotion detection systems in the future. Additionally, cortisol metabolites can be additional biomarkers to stress hormones that increase the efficiency of detecting emotional stress. Besides, antistress hormones can also potentially be used as stress biomarkers. Regarding cortisol detection using biochemical sensors, previous studies have shown three main methods, employing antibodies, aptamers, and MIPs. These methods have significant advantages over blood tests through classical laboratory techniques, as the latter requires a greater number of samples to be taken, consumes significantly more time, and needs trained staff to operate advanced tools (Dalirirad and Steckl, 2019). In comparison between aptamers and antibody methods, aptamers are not rejected by the human immune system, as they are usually not considered foreign bodies, which makes them weakly immunogenic and toxic molecules, unlike antibodies that are highly immunogenic and toxic molecules (Ireson and Kelland, 2006). Additionally, aptamers have more thermal stability than antibodies because of the nature of oligonucleotide-based aptamers, which can maintain their structure, while protein-based antibodies lose their structure at high temperatures. Therefore, aptamers can be used in various assay conditions (Song, Lee and Ban, 2012). Additionally, the production of aptamers is a cost-efficient approach compared to antibody production and allows for easier modification to different chemical reactions (Birch and Racher, 2006; Ferreira and Missailidis, 2007). Lastly, for future stress biomarkers that may have weak immune responses, such that antibodies cannot be generated or produced, aptamers can be recommended, as they can detect ligands that antibodies cannot recognize (Jayasena, 1999). However, in a comparison between MIPS and aptamers, MIPs seem to be more economical (Ramstorm, Ye and Mosbach, 1996) and more specific in terms of target binding (Mugo and Zhang, 2019). Generally, MIPs have advantages over all other recognition systems, as they have high selectivity, are inexpensive, have accurate mechanisms, and are environmentally stable, as can be seen in Table 2. Therefore, due to these advantages, MIPs have been widely used in several industries, including in chemical sensors and drugs (Parlak et al., 2018). However, the detection of cortisol directly from sweat via e-nose technology is under-

researched. In 2009, a study (García-cortés et al.,2009) showed a promising result, here e-nose detected stress situations by measuring the concentrations of cortisol and adrenaline in sweat; however, no further studies have been carried out on this until the pandemic as Durán Acevedo, Carrillo Gómez and Albarracín Rojas, 2021 succeeded to detect stress in the academic context. This study might re-open the gate for further research.

Alternatively, recent studies identified VOCs stress biomarkers emitted from the sweat or the skin during stress events (Martin *et al.*, 2016; Tsukuda *et al.*, 2019). However, the results of the two studies are controversial in many aspects. In the first study (Martin *et al.*, 2016), samples were collected from eccrine glands (foreheads). Four stress biomarkers were found, while in the second study (Tsukuda *et al.*, 2019), no stress biomarkers were identified from the eccrine glands (palms), even though very similar methods (GC/MS) were used. This inconsistency raises the question of whether the eccrine glands are similar in different areas across the body. As some researchers have linked emotional sweating to the apocrine glands (Shelley and Hurley, 1953; Tsukuda *et al.*, 2019), it is also required to know if the eccrine glands produce emotional event VOCs. On the other hand, the source of stress VOCs identified by (Tsukuda et al. (2019) from the axillary area in the study is still unknown. The first possible source assumed was the apocrine gland, while the second possible source was floral bacteria. Addressing this issue may help find answers to the previous questions. Even Gioia et al. (2022) completed their experiment in response to our request, they still need to address the scientific shortcomings raised. The investigation discovered a novel possible biomarker that had not been detected in prior studies but was classed as belonging to the same acidic group. Though some E-noses have been extensively studied and do provide excellent sensitivity to VOCs, they still have drawbacks when used as portable, real-time monitors due to factors like size and power consumption.

With respect to cortisol metabolites, they have been used as additional biomarkers for the stress hormones cortisol and cortisone for more accurate measurement. However, cortisol metabolites are only present 10 min after the production of cortisol in stressful events (Runyon *et al.*, 2019), which raises concerns regarding the effectiveness of utilizing them as biomarkers for acute stress, as this may require an immediate response. They may, however, be useful for less rapid stress situations or chronic stress

conditions in mental health. Another challenge in this regard is that cortisol metabolites respond differently according to each individual, which suggests a need to develop techniques to deal with such individual differences (Runyon *et al.*, 2019). With respect to antistress hormones, they are produced as a response to the production of stress hormones (Lathe, 2002). Oxytocin has been classified as an antistress hormone (Uvnäs-Moberg and Petersson, 2005). Although it has not yet been used as a stress indicator, its presence in biofluids has previously been detected (Mervinetsky *et al.*, 2019). More investigations are needed to check antistress hormone reliability as stress biomarkers, for example measuring the time between the production of stress hormones and antistress hormones. Additionally, although their presence in biofluid has been confirmed, their amounts in sweat must be confirmed in a measurable range.

The literature identifies the cortisol as a major biomarker and, the MIP biosensor technology is the most advanced methodology to detect cortisol. Therefore, the next chapter will discuss the advanced research of MIPs biosensors.

Chapter 3: Molecularly Imprinted Polymers (MIPs)

3.1 Introduction

3.1.1 The concept of MIP sensing technology

In the modern era, ambient environment sensing is widespread. Molecular recognition is crucial to biological processes and is currently the focus of much scientific study due to its importance in catalysis, separations, and sensing processes. While biological systems can generate antibodies against foreign bodies, employing such systems in chemical processes involves many concerns, including cost and environmental sensitivity. Recent efforts in sensor research have focused on creating artificial receptors that can detect and respond to targets with the same specificity and sensitivity as antibodies and antigens. When combined with modern techniques for monitoring changes in the recognition elements, this molecular recognition effectively produces selective, sensitive sensors of non - invasively detecting and monitoring targets (Belbruno, 2019).

MIPs are synthetic versions of the physiological antibody-antigen system. As such, they use a "lock and key" mechanism to selectively bind the molecule with which they were produced. As a result, MIPs have the potential to provide the specificity and selectivity of biological receptors with the added benefits of environmental durability and low cost. Natural receptors, for example, typically require storage and application at temperatures in the human body temperature range. In contrast, MIPs based on a polymer host can typically be stored indefinitely, do not require special environmental storage conditions, and can be applied over a much more comprehensive temperature range (Belbruno, 2019).

Multiple production methods will be discussed in a later section of this chapter. However, they all follow the same basic outline: A three-dimensional polymer network formed by a combination between an analyte, also known as the template, and functional monomers within the company of a cross-linker agent. After that, the analyte is extracted from the polymer to leave recognition sites integral to the template molecule in terms of shape, size, and chemical functionality (Turiel and Martín-Esteban,

2010). Another advantage of synthetic receptors is their near universality, especially for tiny compounds. In contrast to biological systems, where the target must match an available antibody or be uniquely synthesised for that target, MIPs may be created for practically any target molecule. In terms of cost, MIPs are often less expensive than natural antibody prices.

3.2 MIPs Production

Because of the widespread application of MIPs technology, researchers have devised multiple strategies for preparing MIPs, each tailored to take advantage of the unique characteristics of the rigid polymer. Synthesis, phase inversion, and soft lithography are the three basic techniques used, as seen in figure 6 (Belbruno, 2019).

Covalent, non-covalent, and semi-covalent are the three described preparation methods for synthesis, the most prevalent technique. Regarding the covalent method, reversible covalent bonds are pre-polymerized between the analyte and monomers. The matching covalent connections are then broken to extract the analyte or template from the polymer. High contact stability between the template and monomers promotes the homogeneity of specific sites and decreases the prevalence of non-specific sites (Wulff and Sarhan, 1972). However, as the formation of covalent bonds and the removal of the template are easily modifiable, this method has significant limitations when it comes to generating suitable template monomers under complex mild conditions (Turiel and Martín-Esteban, 2010).

Semi-covalent refers to a method used in molecular imprinting, where the template molecule is covalently attached to a functional monomer, but the rebinding of the template is dependent only on non-covalent interactions (Sellergren and Andersson, 1990). The non-covalent method depends on the development of a weak bond between the analyte and the monomers. According to research, this is the most common method for manufacturing MIP (Arshady and Mosbach, 1981; Chen et al., 2016).

In the method of synthesis production, a functional monomer interacts with the target molecule in solution to promote a network of covalently or noncovalently interacting complexes. Thus, functional monomers such as methyl methacrylate (MMA) and methacrylic acid (MAA) provide a reactive substituent to begin a covalent interaction

with the template. Following the addition of a cross-linking agent and polymerization initiator, heating or UV-driven polymerization is initiated. The final step of the interaction process is the generation of a powder suitable for separation. After covering the substrate with the reaction mixture and initiating the polymerization by photoinitiation, a film rather than a powder can be formed. Notable functional monomers include methyl methacrylate (MMA), acrylamide, and methacrylate acid. Ethylene glycol dimethacrylate (EGDMA) is the most often used cross-linker (Jafari, Rezaei and Zaker, 2009).

Phase inversion is processed in the absence of a cross-linker, which causes less homogeneity of the binding sites. Instead, the host polymer and its template are bound together by adding a poor solvent, resulting in MIP as a membrane or beads (Belbruno, 2019).

Finally, soft lithography is a process that blocks the sensing large molecules from passing into the cavities under the surface by producing a surface- imprinting materials that are applied to them. The simple model of this process utilizes the fabrication of a stamp made from a self-assembly array of the template. Then, the template is compressed to a semi-polymerized film and maintained until the polymerization of the film is fully completed (Odom *et al.*, 2002; Qin, Xia and Whitesides, 2010).

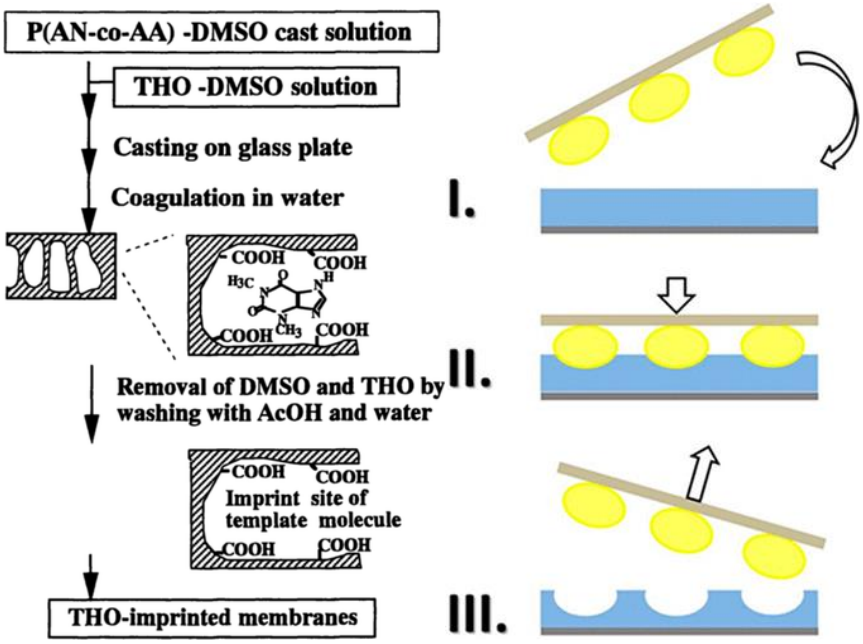
In terms of extraction methods, several purification formats of the technical applications of MIPs have been utilized for expected intrusions removal, adjustment of the analyte form, and providing the reproducibility option apart from the variation of the sample matrix (Smith, 2003). However, molecularly imprinted solid-phase extraction (MISPE) is the most sophisticated technology application of MIPs since it allows users to adjust the sample treatment step before commencing the final step. Furthermore, a rising range of sample preparation procedures, such as solid-phase microextraction (SPME) and matrix solid-phase dispersion (MSPD), have been integrated with MIPs (Ramos and Smith, 2007; Pedersen-Bjergaard and Rasmussen, 2009).

Furthermore, following polymerization, the CV electrochemical extraction method is also presented. By repeatedly applying cyclic voltammetric scans, the approach aims to breakdown the bindings between the bonded targets and the cavities (Mugo and Alberkant, 2020). Consequently, our work simulates the CV extraction approach utilizing

a function generator to clean the sensor following an actual binding. Such a process can be a part of the charging process of a smartwatch.

Several parameters must be taken into consideration while using MIPs based biosensors. First, the imprinting factor (IF) can be defined as the proportion of the analyte's binding in the imprinted polymer to the binding of it in non-imprinted polymers. Second, the binding capacity (BC) is the percentage of dividing the concentration of the target molecule adsorbed from the test solution by the initial concentration of that solution. Last, but not least, the response time (RT) the definition of this parameter is controversial as it has been defined differently by several authors. The model definition is the time needed to complete 62.3 per cent of the last signal beginning from when the stimulus is applied (Belbruno, 2019).

A) B)



C)

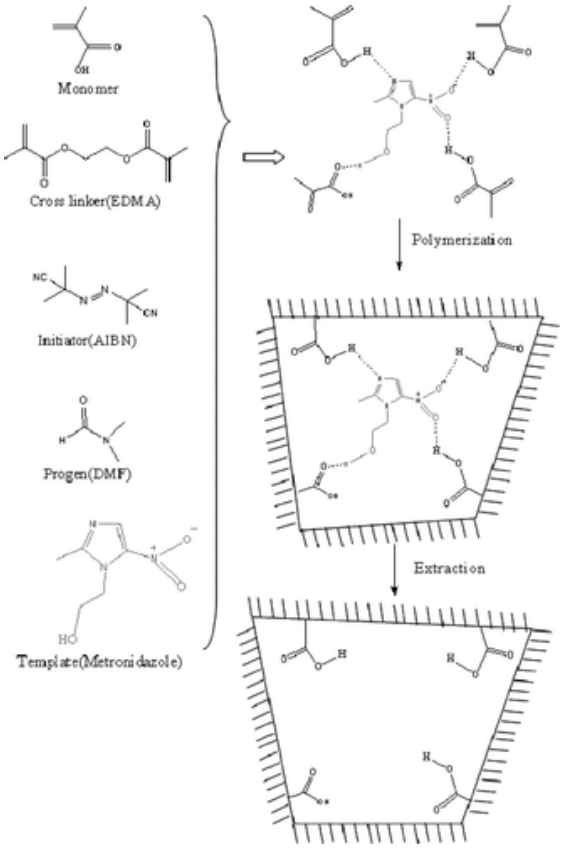


Figure 6

A) This diagram illustrates the process of creating molecularly imprinted polymers (MIPs) using phase inversion when water is used as a template. Typically, template

stamps are created on polymer film by self-assembly before they are prepared for molecularly imprinted polymers (MIPs). For the stamp to produce desired effects, it is pressed onto the film during polymerization. B) Molecularly imprinted polymers (MIPs) are prepared using soft lithography or surface stamping by creating polymer film templates, typically assembled by self-assembly techniques. It is then possible to extract the stamp from the polymerized film, which creates binding cavities corresponding to the template, and then press it onto the polymerized film. C) Molecularly imprinted polymers (MIPs) are produced by combining the template molecule with a functional monomer capable of forming hydrogen bonding interactions and a porogen solvent. To initiate polymerization, cross-linkers and initiators are introduced into the mixture, completing the polymerization process (middle right). In the final step, the template molecule is extracted using an appropriate solvent, which results in the formation of the MIP with binding sites available for subsequent rebinding (Belbruno, 2019).

3.3 MIPs sensing techniques.

Methods for sensing MIPs include electrical (Chemiresistors, Capacitance Sensors, Field Effect Transistors, and Electrochemical Impedance Spectroscopy), Quartz Crystal Microbalance (QCM) Sensors, Electrochemical Sensors, and other sensing technologies like MIP-coated Surface Plasmon Resonance (SPR) crystals and MIPs optical biosensors (Belbruno, 2019).

In our research area, a MIPs electrochemical biosensor is a powerful instrument for detecting cortisol in sweat. In this regard, a brief review of the fundamentals of this sensing approach will be discussed. Typically, three-electrode electrochemical MIPs biosensors are used to analyze liquid samples. In these biosensors, the current is monitored in two ways: amperometrically, when the potential is held constant, and potentiometrically, when the potential varies. However, both approaches have some common ground despite their obvious differences.

3.3.1 Conductometric and impedimetric

This approach relies on the time monitoring of the ionic changing at the solid-liquid interfaces resulting from the conductivity of the MIP receptor layer with the binding of a target analyte (Sergeyeva *et al.*, 1999). Typically, electrochemical impedance spectroscopy (EIS) is used to measure the ionic change. EIS analyses the

impedance of a system apart from the impedance of the alternating current, assessing the capacitive and inductive effects (Grieshaber *et al.*, 2008). Recently, EIS has been considered one of the most powerful analysis tools and widely used in either on vivo studies (Wackers *et al.*, 2020) or in vitro studies (Cai *et al.*, 2010).

3.3.2 Voltammetry and amperometry

Voltammetry is an interesting approach for sensor engineers wanting to fabricate highly sensitive bio(memetic) biosensors due to their versatility and low noise (Su *et al.*, 2011). This method involves three electrodes: the working electrode, the counter electrode, and the reference electrode, with an aquatic electrolyte inside a defined electrochemical cell. In a defined range, the voltage is swept, resulting in a current monitored in time regarding raising the concentration of the target analyte. In the case of increasing the peak of the current by several voltages, multiple target analytes can be detected. Voltammetry is suitable for biofluidic samples and does not require costly metal, as graphene electrodes are utilized in numerous applications (Cheng *et al.*, 2020). Furthermore, electrochemical interferences can be prevented by several methods like Voltammetry and amperometry (CV), differential pulse voltammetry (DPV), and square wave voltammetry (SWV), resulting in increasing the sensitivity to the analyte of interest (Zhang *et al.*, 2020).

Amperometry approaches are a subset of voltammetry approaches that operate at a fixed optimal potential. At the operation potential, the target analyte is surrounded by the MIP and either reduced or oxidised, providing an electrical current suitable for the analyte of interest (Apetrei and Ghasemi-Varnamkhasti, 2013).

3.3.3 Field-effect transistors

These transistors typically contain three conducting electrodes: the drain, the source, and the gate. The first two electrodes are used to segregate the biosensor's semiconductor path, whereas the third is utilized to provide a bias voltage across the sensor. The semiconducting path between the source and drain is guarded by receptor layers affixed to an insulating layer, such as polymers or metal oxidation. When the recognizing layer captures the target, the charge density at the liquid-solid interface alters the conducting channel between the drain and source. The change of the conducting channel either increases or decreases depending on the specific design of

the sensor and the target properties, which may be monitored electronically (Chunta, Suedee and Lieberzeit, 2018).

3.4 Capacitive sensing

Capacitive sensing technology has been used widely in a growing number of industries as it offers an economic property in manufacturing and energy consumption (Braun *et al.*, 2015) and flexibility in fabrication design (Ye *et al.*, 2020). Based on its principles, Capacitive sensing technology can be a tool to measure any objective that is either conductive or has a dielectric constant non-identical to the air value (Baxter, 1997). First, the capacitance technology is divided into three primary modalities, volume sensing; in this model, the measured capacitance varies with the object's physical characteristics. It is a dielectric constant or the change of the electric field self-generated by the object. This modality is represented in several applications, including capacitive gas sensors (Pourteimoor and Haratizadeh, 2017) and capacitive voltage sensors (Zhu, Lee and Pong, 2017). The second modality is deforming sensing; Here, the deformation of the electrode causes a change in capacitance. Thus, it is common to use it in measuring physical quantities such as angles (Fulmek *et al.*, 2002) and force (Jindal *et al.*, 2018).

Finally, displacement sensing measures the change of the capacitance once the object has moved a distance from the electrode. This technique is used in motion centred applications such as location and movement tracking(Arshad *et al.*, 2017) as well as in human-computer interaction systems (Mühlbacher-Karrer *et al.*, 2017).

3.5 Capacitive electrochemical biosensors

In real-time, capacitive biosensors measure the change in binding capacity between the target molecule and the sensor surface. Thus, this straightforward design can be an efficient alternative to the prevalent ELSA immunoassay method (Mattiasson, Teeparuksapun and Hedström, 2010). In detail, during an event of analyte-reception interaction, the capacitive biosensors register the change in the dielectric properties. Also, it might register the change in the thickness of the dielectric layer (Berggren, Bjarnason and Johansson, 2001). The binding event displaces an ion around the capacitive electrode, leading to capacitive reduction. The higher displacement of ions, the more significant decrease in capacitance is registered

(Mattiasson and Hedström, 2016). Due to the simplicity of this method, researchers developed a variety of capacitive biosensors to detect various targets, including Protein detection (Labib *et al.*, 2009; Loyprasert *et al.*, 2010; Teeparuksapun *et al.*, 2010), Nucleic Acid detection (Park *et al.*, 2012; Thipmanee *et al.*, 2012; Tsekenis *et al.*, 2012), Cell detection (Brzozowska *et al.*, 2015; Rocha *et al.*, 2016; Rydosz *et al.*, 2016), heavy metal (Corbisier *et al.*, 1999), glucose (Labib *et al.*, 2010), and molecules (Labib *et al.*, 2010). Additionally, because MIPs technology can mimic the natural recognition cavities, which can also modify shape, size and position, more applications can utilize the technology compared to the natural electrochemical methods (Cieplak and Kutner, 2016). Moreover, this study's fabricated sensor has used this capacitive method in different research (Mugo and Alberkant, 2020; Mugo et al., 2022; Mugo, Lu and Robertson, 2022).

3.5.1 Electrochemical impedance spectroscopy

Against this background, impedance change also has been utilized in capacitive MIP biosensors research. This method is sensitive to surface phenomena and changes in characteristics, making it an ideal tool for evaluating the platform's sophisticated electrical resistance. It is, therefore, a crucial method in electrochemical studies (Lisdat and Schäfer, 2008).

Impedimetric sensors are bioelectronic devices that rely on biomolecular interactions with a conducting transducer surface. The detection technique requires developing a recognition combination at the interface between the sensing biomolecule and the analyte, which modifies the electrical characteristics of the recognition surface (Lisdat and Schäfer, 2008).

Instead, the interaction with an analyte molecule can be monitored by immobilizing the biological component on the working electrode. In this case, the impedance of the sensing working electrode determines the overall impedance (Liu *et al.*, 2006). Impedance spectroscopy is mainly used in bioanalytics to monitor the biosensor fabrication process. It is, however, also utilized to examine the biosensor's recognition process. Proteins, biopolymers, cells, microorganisms, antibodies, and antigens are just a few of the (bio)analytes that have been demonstrated to have applications (Katz and Willner, 2003).

The capacitance of the electrochemical doubling layer is affected by all substances present within the biosensor interface, primarily solvent molecules, immobilized biomolecules, and coatings that enhance immobilization and detection. Variations in capacitance are caused by changes in the dielectric constant or the thickness of the double layer on the transducer's surface (Berggren, Bjarnason and Johansson, 2001). When designing a capacitive biosensor, the electrode surface is typically coated with an extra insulating layer to minimize Faradaic currents. On top of this layer is immobilized the biorecognition element (Liu *et al.*, 2006). The effect of the electrical field on the biological recognition event can also be studied by varying the working potential used in conjunction with capacitance measurements taken in the absence of Faradaic currents (Bart *et al.*, 2005). Figure 7 shows how a biosensor is developed by immobilizing the recognition element on a single working electrode. Recognition activities at the sensor surface can be evaluated in several ways, including directly detecting the overall impedance at specified frequencies and analysing changes in electrode capacitance or resistance (Liu *et al.*, 2006).

MIPs generate a surface that functions as the analyte's negative. Therefore, they can be utilized to determine the analyte in a solution. Due to their chemical composition, they may be recreated in various ways and are stable for prolonged periods in several different conditions. In addition, thin surface coatings are appropriate for impedimetric binding event transduction. Consequently, capacitive sensors for organic substances have been developed (Liu *et al.*, 2006). Biological material's impedance is analysed as a function of a particular analyte's concentration in solution. This is comparable to an investigation of solution resistance as a result of ion concentration (Cheung, Gawad and Renaud, 2005). The rising cell density within the solution causes a change in impedance that is clearly observable (Liu *et al.*, 2006).

In detail, binding the target molecules with the cavities causes changes in the MIP layer's electrical properties on the transducer surface, including its conductivity. The higher concentration of target molecules, the more remarkable change in conductivity response. As a result, it increased in capacitance in parallel with the increase in conductivity (Asghar *et al.*, 2019). They connected a glucose MIP biosensor to an LCR meter and added several glucose concentrations to the MIP surface. The higher the response, the higher the concentration sample (50ppm) as the capacitance was raised

to 393nF. In contrast, one ppm was only 9.99nF. Our research implements a similar method.

Storer et al. (2018) investigated the strong binding of a three-phosphate MIP formulation against nitrate, sulphate, and NIP. The result of the study shows a significant response of the MIP1 to phosphate compared to nitrate and sulphate. Moreover, all three MIPs have higher responses to phosphate than NIP.

Herein, as our biosensor was only used with the traditional electrochemical capacitive biosensing using the CV method, we found that using the impedance analysis capacitive biosensing technique, LCR meter, is under-researched. Therefore, our experiment using LCR will be presented in Chapter 4, and its results will be discussed in Chapter 5.

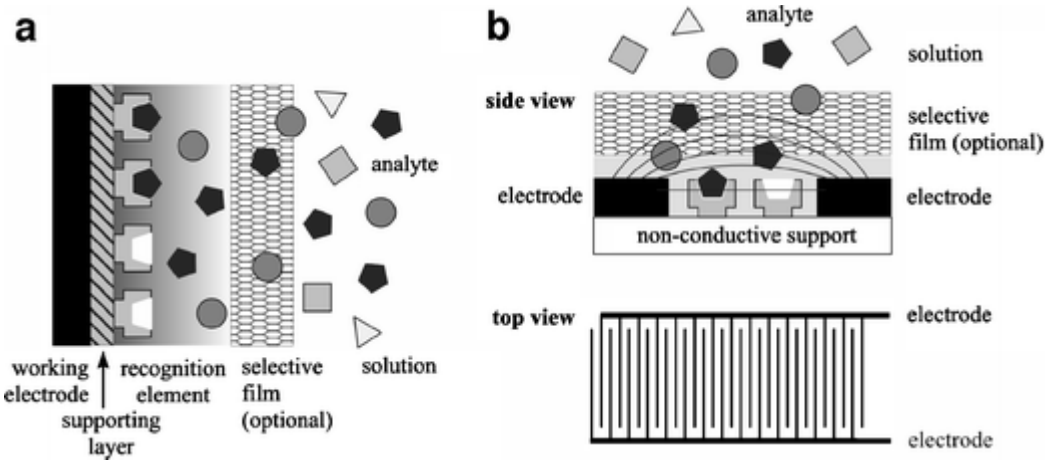


Figure 7 Designs for impedimetric biosensors that function on a modified working electrode (a) or an in-plane arrangement of two electrodes (interdigitated electrodes, IDE) (b). A biocomponent is immobilized on the electrode in a and between or on the electrodes in b. ***(Liu* et al*., 2006)***

3.6 Cleaning MIP sensors

In the meantime, using electrochemical biosensors for long-monitoring applications is a complex challenge. Even though the importance of these sensors as powerful tools for the analysis of a wide range of targets, the electrochemical removal of the targets and the interfering molecules, such as proteins, electrolytes, and glucose, are significant factors that reduce the sensitivity and the lifetime of the electrode (Goda *et al.*, 2016; Li *et al.*, 2016; Xu and Lee, 2020). In addition, the literature found that after sweat detection cortisol sensors can become contaminated with perspiration and are

not generally reusable due to the fact that sweat degrades electrode surfaces (Sekar *et al.*, 2020).

As a result, several treatments or even re-build the sensing layers must be applied to refresh the electrode for the electro-active species. In terms of the electro-inactive species targets, researchers have tried different techniques to address this problem, such as developing label-free biosensors and utilizing disposable screen-printed electrodes (SPE). However, in both scenarios, the problem is far from resolved (Zhao *et al.*, 2015; Xu and Lee, 2020).

In recent years the development of self-cleaning electrochemical biosensors and antifouling techniques has been an attractive area of research. Lin and Li (2020) reviewed antifouling strategies in electrochemical sensors, including surface antifouling mechanisms and electrode modification strategies. In terms of Surface antifouling strategies, four main strategies have been presented: hydration layer, steric repulsion, electrostatic repulsion, and surface topography. On the other hand, the modification strategies for antifouling electrodes are divided into two main strategies: physical and chemical. The former ones often do not change the chemical properties. The first strategy is physical adsorption, and it has a constant cover of the materials with a blocking layer to decrease the direct connection between foulants and electrodes. The second is the mechanical coating. In this method, polymers and films coat the antifouling reagents. The third is the superhydrophobic strategy, which is based on engineering the surface of electrodes without alteration of the chemical properties; they divided into two main strategies; the self-cleaning strategy, which is inspired by the lotus leaves an effect, building a superhydrophobic surface that causes water to glean impurities and prohibit any molecular attachment (Hang *et al.*, 2019). The second hydrophobic strategy is constructing the electrode surface's morphology with a unique structure that prevents the direct contact of cells and proteins.

Last, but not least, regarding the nanoporous structure, recent studies show a proper electrode topography can increase the antifouling (Lin and Li, 2020). Furthermore, nano porous electrodes have been revealed to perform better in minimizing biofouling than planar electrodes due to the porous interior structure limiting the mass transport speed and minor molecules from an effective exchange (Sang, Vinu and Coppens, 2011).

The chemical strategies present the popular self-assembled monolayers (SAMs) and Polymer brushes (Lin and Li, 2020). Zhang and Chen (2019) classified the strategies of

no-wash electrochemical biosensors into four strategies: Signal change, radiometric assay, direct reaction, and others which are less commonly used strategies in research. The primary aim of the signal strategy is to amend the signal-to-noise ratio (SNR) as much as achievable. The radiometric essay differs from the signal change method, which depends on a single signal. It is designed from a built-in self-calibration of signal intensity, recording a minimum of two targets-induced signal fluctuations (Huang *et al.*, 2018). Both strategies' signals are generated from the electrochemical activities of the functional materials or the biological species, unlike the direction reaction method that involves the target analyte directly to the reaction to obtain a specific electrochemical signal. This can be done by either obtaining the signal generated from the target analyte's direct reaction or the target's induced reaction. Xu and Lee (2020) classified the antifouling strategies for the implantable biosensors into two categories: the passive antifouling strategies, which include hydrophobic materials, PEG, hydrogels, Zwitterionic Polymers, bio-mimicking materials, superhydrophobic surfaces, and drug-eluting materials, and active-biofouling strategies that include, temperature, responsive, surfactant desorbing surfaces, acoustic waves, and magnetic actuation. Most recently, Cui et al. (2022) developed a novel dual-responsive electrochemical biosensor by combining artificial protein MIPs and natural hyaluronic acid (HA) probes on a flexible electrode to detect the cancer biomarker Cluster of Differentiation 44, CD44. Both types of probes have been thoroughly examined and compared regarding their effectiveness. A new design for screen-printed electrodes (SPEs) included dual channels, where one channel utilized protein MIPs synthesized through a polymerization process involving alginate gel, a biocompatible antifouling chemical. In the second channel, natural HA probes were immobilized. Due to the use of this dual-channel approach, the quantification of targets was highly sensitive and selective, with a LOD of 1.41. Hu et al. (2022) developed an electrochemical antifouling ratiometric sensor for *Zearalenone* ZEN detection. The sensor utilized a BP-graphene oxide (GO) composite, and ionic liquid IL doped MIP, which provided a large surface area and improved electrocatalytic properties for enhanced sensitivity. By preventing nonspecific protein adsorption on the sensing interface, the doped IL in the MIP enhanced selectivity. Fei et al. (2023) demonstrated the importance of antifouling in assembling metal-organic framework (MOF) microcrystals into macroscopic membrane structures. As a result of the use of graphene oxide (GO) as a nano-surfactant, super hydrophilic

amino acid-based Zr-MOF microcrystals (MIP-202(Zr)) were successfully dispersed and incorporated into a bulge-structured membrane. The resulting GO/MIP-202(Zr) membrane exhibited enhanced water permeance, superior to pure GO membranes and other GO/MOF composites. The membrane also demonstrated satisfactory rejection performance for dye molecules, depending on their size, charge, and configuration. Further, GO/MIP-202(Zr) membranes demonstrated improved antifouling properties in comparison with pure GO membranes, which demonstrated the membrane's potential for practical separation processes as well as provided insights into the assembly of MOF microcrystals into macroscopic membranes for water treatment.

As practical examples of self-cleaning biosensors, Wei et al. (2018) fabricated a self-cleaning electrochemical protein imprinted biosensor based on thermoresponsive memory hydrogel applied on a glassy carbon electrode with a polymerization method to self-clean bovine serum albumin (BSA) in aqueous media. The sensor cleaning method involves potential cycling between -0.8 V to 0.8 V for 14 cycles at 37 centigrade degrees in a Phosphate-buffered saline (PBS) buffer with no cleaning solvents. After the cleaning, all the BSA molecules were removed without any changes in the structure, and the electrode was ready for further detection. Hang et al. (2019) developed a self-cleaning Hierarchical graphene/nanorods electrochemical biosensor to detect H2O2 in blood serum. The biosensor consists of ZnO nanorods split on vertical graphene nanowalls (VG/NRs) and fluorinated to present the antifouling properties. The sensor shows high sensitivity in water and serum as well as excellent antifouling due to the significant level of hydrophobicity. Zhang, Wang and Li (2019) developed a self-clean electrode with an association of ratiometric electrochemical strategy to simulate the detection of adrenalin, serotonin, and tryptophan. A glassy carbon electrode is modified using PDMS as a hydrophobic layer combined with the zeolite imidazole framework (ZIF) to obtain self-clean electrodes. Unlike the traditional ratiometric method, which modifies the probes on the electrode surface, the novel strategy is conducted by adding the internal reference probes directly into the electrolyte solution. Zhu et al. (2017) fabricated a self-clean working electrode to eliminate unspecific adsorption of molecules using hydrophobic and conductive nanocomposites of PDMS and multi-walled carbon nanotubes to modify glassy carbon electrodes. Therefore, the electrode has self-clean properties because of the hydrophobicity and the electron transfer

capability due to the conductivity. Interestingly, Manickam et al. (2017) developed a self-clean MIPs biosensor to detect cortisol in saliva samples by the electrochemical over-oxidization process. This process removes all the bound cortisol molecules from the polypyrrole (PPy) matrix scanning the potential in a range of 0 V to +0.8 V for 25 cycles in PBS solution at a scan rate of 50 mV/s. A broad irreversible voltammetric peak was observed at approximately 3V during the elution scan because of the over-oxidation of PPy. On the other hand, no cathodic peaks have been observed during the reverse scan, which indicates the electrochemically irreversible properties of the over-oxidation of the PPy process.

Template removal is a crucial step in the fabrication process of MIP. At this step, templates are extracted from the polymer to create cavities that rebind with the targeted template molecular. A wide range of techniques have been used at this stage, and these techniques are classified into three general methods: Solvent extraction, physically assisted extraction and supercritical solvents (Lorenzo *et al.*, 2011). CV is a standard method to extract cortisol templates in cortisol MIP biosensors. Bian et al. (2022) performed 20 cycles to break the hydrogen bonds between the cortisol templates and polypyrrole, resulting in cortisol cavities in the MIP film.

3.7 Adjustments of MIPs for wearables

3.7.1 Cyclic Voltammetry

In electrochemistry, voltammetry-based electroanalytical methods are commonly employed to collect quantitative data on redox processes in the lab for real-time monitoring (Stradolini *et al.*, 2017). A voltage sweep is applied between the electrochemical cell's working electrode (WE) and its reference electrode (RE) to excite the cell. The current generated by the oxidation or reduction of the target species is then measured at the counter electrode (CE) or, alternatively, at the working electrode (WE). Current is typically reported versus the electric potential to produce a voltammogram (Carrara, 2013) .

Palmsens 4 potentiostats with PSTrace software may be used for capacitance measurement in the current investigations as an external device to perform CV (Mugo and Alberkant, 2020). On the other hand, such a device may restrict the practicality of

wearables. Also, having a printed circuit board that CV runs for wearables are still in the developing stage (Crozatti et al., 2013). In electronic devices, however, simple LCR meter circuits are produced (Georgas *et al.*, 2022).

To overcome the limitations of the CV methods, our research examined the change in the capacity of the sensor in the addition of cortisol using an LCR meter to characterize the sensor and a signal generator to create the CV signals for cleaning.

3.7.2 Placement of the detection

The presence of eccrine sweat glands at densities of >100 glands/cm2 2 in numerous places of the body enables multiple valid sampling sites. Consequently, the case for utilizing a wearable device to sample and detect sweat is noticeably clear. The advantages of sweat for non-invasive biofluid access are that most sampling sites and surface areas are external to the body, and continuous access is provided. It can be triggered on demand by employing local iontophoresis. It can be sampled at meagre fluid generation rates without external contamination; can be detected before analytes are degraded (Heikenfeld, 2016). Therefore, eccrine glands have been chosen as the intended site of sampling for our constructed device. It has also been designed into the wristband to be within easy reach. Also, having a wristband wearable delivers visual feedback on the user site. In addition, it allows for future adaptability when used with multi-model biosensors.

3.8 Conclusion

This chapter summarizes the history of MIPs and their uses in many industries, which led to the development of various production techniques. The extraction procedure removes the artificial targets to create the binding sites and is a crucial component of MIP manufacture. However, this study indicated that MIPs utilized in studies are discarded after single or multiple applications. Thus, we discovered that repeating the extraction process to eliminate the bound targets following the experimental trials has received insufficient attention.

In addition, the chapter provides an overview of the sensing technologies associated with MIPs. The chapter's primary focus was capacitive MIPs and the most recent capacitive sensing techniques employed in the field. Capacitive biosensing in MIPs LCR meter approach is under-researched, although CV methods are extensively utilized in electrochemical detection. For example, the literature indicates that measuring

cortisol in wearables must be paused for CV scans between measures. In comparison, an LCR meter may offer continuous measurement and real-time monitoring. Additionally, it may be more functional for wristband design.

This research indicates that the impedance change measurement meter is the most suitable capacitance approach for the real-time monitoring of wearable devices. In the lab, spectroscopy can be characterized with a bench LCR meter. A wearable device would implement to spectroscopy method using the microprocessor of the wearable device.

Therefore, in the following chapter, the research will evaluate capacitance measurement utilizing an LCR meter on MIPs produced for use with CV approaches. In addition, the literature uncovered the MIP's need for reusability. Therefore, this study will mimic the CV extraction approach using function generators so that it is suited for wearable devices in the following chapter.

Chapter 4: Methods

4.1 Introduction

Our MIPs biosensors were designed as part of this research and kindly manufactured by Dr Samuel Mugo's team and in Canada. Everything required to manufacture the imprinted polymers was purchased, as specified in the previous research (Mugo and Alberkant, 2020) as following: All aqueous solutions were prepared using > 18 MW Milli-Q water (Millipore, Bedford, MA, USA). The Dow and Corning's Sylgard 184, polydimethylsiloxane (PDMS) was purchased from Dow Corning Corporation, Midland, MI, USA. The Ecoflex 00–30-part A, part B was bought from Smooth-on Incorporation, Edmonton, Alberta, Canada. The multi-walled carbon nanotubes carboxylic acid functionalized (diameter 9.5 nm × 1.5 µm), hydrocortisol, cyclohexanol, glycidylmethacrylate (GMA), potassium ferricyanide, ethylene glycol dimethacrylate (EGDMA), (3-glycidyloxypropyl trimethoxysilane) (GOPS), and 4,4'-azobis (4-cyanovaleric acid) (ACVA) were obtained from Sigma-Aldrich, Oakville, Ontario Canada. The cellulose nanocrystals (CNC) were donated by Alberta Innovates, Edmonton, Alberta.

However, our modified biosensor was tailored to our needs. The most recent cortisol MIP biosensor consisted of 400 µL of cyclohexanol, 26 µL of GMA, 80 µL of EGDMA, 2.0 mg of ACVA, and 1.5 µM of cortisol dissolved in a 1/1 (v/v) mixture of water and acetonitrile (Mugo and Alberkant, 2020). However, previous research indicates that the optimal ratio of cortisol to polymers is 1:5 (Ramsttrom, Ye and Mosbach, 1996). In addition, the 1:6:30 Molar ratio of template/functional monomer/cross-linker (Parlak et al., 2018) and dichloromethane (DCM) showed better performance than acetonitrile to make the polymer more porous and, as a result, more sensitive, by providing more binding sites (Ramstorm, Ye and Mosbach, 1996). Therefore, our batch of MIPs sensors was specified based on the mentioned details. Also, two different batches of MIP have been utilized, which have differences. The main difference is that the second batch has a higher cortisol response and is more hydrophobic. In contrast, the second batch responds faster as the surface gets saturated faster. All electrodes were connected by winding conductive wire around the electrodes to avoid any damage caused by direct connection with mechanical clips as can be seen in Figure 8.

A) B)

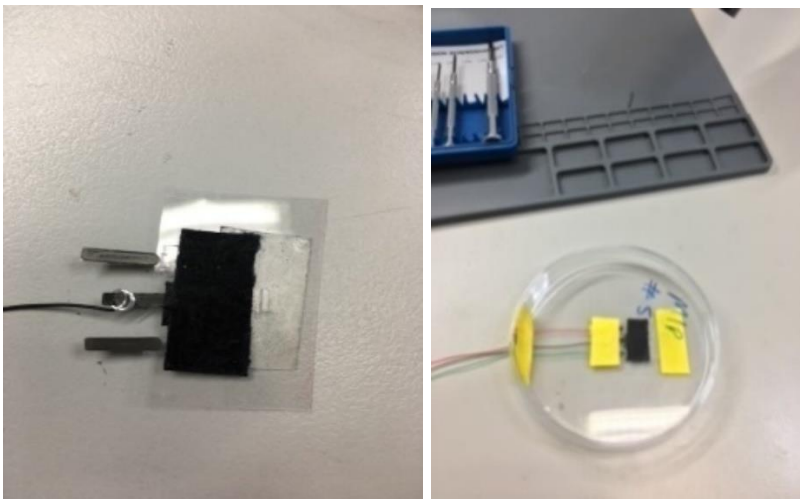


Figure 8

A) depicts a wire wound electrode attached to the LCR meter clips. Other electrodes follow the same procedure. B) depicts the biosensor's final design, with the reference electrode in red, the working electrode in black, and the counter electrode in green.

4.2 Materials and Methods

4.3 Devices

4.3.1 The LCR meter

The LCR meter used in our experiments was the LCR-6002 (GW-instek). LCR meters measure the capacitance and resistance of electronic components and circuits. These properties are characteristics of the device or circuit related to its electrical behaviour and are typically expressed in Farads (f) and Ohms units, respectively. To measure the response of an electronic device or circuit to changes in concentration using an LCR meter, it would need to experiment with varying the concentration of a solution and measuring the resulting changes in the capacitance and/or resistance of the device or circuit. The LCR meter is connected to a laptop, and all the results of the tests will be extracted in an excel sheet using putty software.

4.3.2 Function generator

The device used to generate the required functions was the TG2001 (Thandar). It is an electronic device that generates a spectrum of electrical waveforms at different frequencies. These waveforms can be used to test and validate electronic circuits and systems.

4.3.3 A digital storage oscilloscope (DSO)

The experiments used the TDS 1012B (Tektronix) two-channel digital storage oscilloscope (DSO). The purpose of a DSO is to measure and analyse electrical signals that are going to be used through the function generator. It has a display screen and a system for capturing and storing waveform data. A two-channel DSO can measure and display two distinct input signals simultaneously, allowing the user to compare and analyse the signals side by side.

4.4 Chemicals

Our Cortisol was purchased from Sigma Aldrich (Hydrocortisone (H4001)) and Phosphate buffer (Phosphate buffer pH 7.2 at 25 °C (P3288-1VL)), (Merck LTD, Floor 4, Merck House, Seldown Lane, Poole, UK). The Artificial sweat (Artificial Sweat without customization, NCZ-APS-0011-20) was bought from (NANOCHEMAZONE™, Edmonton, Alberta, Canada).

4.4.1 Cortisol preparation

To prepare our stock cortisol, 25mg of cortisol is added to 100 ml phosphate buffer for 0.25 mg/ml of cortisol. Then different dilutions have been made out of this stock. including 75000 ng/ml, 50000 ng/ml, 25000 ng/ml, 140 ng/ml, 100 ng/ml, 60 ng/ml, and 20 ng/ml. As the physiological range of cortisol in human sweat is between 8 and 140 ng/ml). Initially, 75000 ng/ml and 5000 ng/ml were utilized for confirming the detection, and as stock for dilutions.

4.4.2 Preparation of 0.1M Phosphate buffer.

0.71g of Phosphate buffer pH 7.2 stock have been added to a final volume of 50 ml of distilled water.

4.4.3 Fabrication of the capacitive sensor

The biosensor is manufactured on a patch (made from PDMS) that was created in four steps: 1) PDMS was initially produced by combining a cross-linker and a curing agent. Ecoflex is then created by combining Ecoflex elastomer and hardener. 2) The two mixtures were then combined. 3) The mixture of PDMS and Ecoflex was then baked for four hours at 72°C to produce a PDMS/Ecoflex micro-pillared patch. 4) After that, silver nanoparticle aqueous suspension was coated on the cured patch, followed by (3-glycidyloxypropyl trimethoxysilane) GOPS supplemented with glacial acetic acid. Regarding silver nanoparticles film, The MIPs polymer was set and dehydrated. The polymer constituted of cyclohexanol, GMA, EGDMA, ACVA, and 1.5µ M of cortisol dissolved in a mixture of water and acetonitrile. Then, the MIPs were polymerized are 70 °C for 4 hours in the oven. Regarding our sensor, a MIP template was extracted using the CV cleaning technique. The MIP films were immersed in 10 mL of 0.1 phosphate buffer in an electrochemical cell connected with reference and counter electrodes. They conducted five CV cycles with potential ranges of +0.9V and -0.9V, and they changed the buffer between every five cycles for a total of 15 cycles. More importantly, the research indicates that the captured analyte can be removed from the cavities through the CV method. Therefore, our work simulates the conditions of the extraction method using a function generator. First, the biosensor is immersed in 10mL of the buffer. Then, the potential range +0.9V –0.9V in a triangle waveform signal was applied to sensor's the reference and the counter electrode.

The biosensor was evaluated by generating cyclic voltammograms in the range of 0.95 V to + 0.95 V at a scan rate of 0.10 V/s (Mugo and Alberkant, 2020). Cortisol concentration was estimated using cyclic voltammograms by calculating the ratio of current to scan rate. There are three electrodes in the CV acquisition system, and the working electrode is presented as-fabricated capacitive Sensor. The auxiliary electrode is platinum, and the reference electrode is Ag/AgCl electrode. As 50 µl of 0.1 M phosphate buffer was placed on the Sensor, 10 µL of cortisol standard at 150 ng/mL was progressively spiked. With every addition of the buffer, cyclic voltammograms were obtained in triplicate. The same test also has been done with a real sweat collected from a sample after an intensive workout session. It is worth presenting that the sensor has also been used to detect other biomarkers in sweat, such as adrenaline and glucose. In this experiment, MIP was compared with a non-imprinted Polymer (NIP), and it found that MIP has higher selectivity and responsiveness than NIP. Also, raising cortisol concentration yielded a linear calibration (R^2 0.92) and increased the isolating properties of the MIP, reducing the capacitance with concentration (Mugo and Alberkant, 2020).

Both MIPs and NIPs were received from our partners in Canada. However, the responses and hydrophobicity of the two batches were very different. Because of this, there is a clear distinction in the results of the tests. A smaller quantity of liquid was required to spike the initial batch. However, the second batch is more responsive than the first, and the reaction due to any liquid is much lower in the first batch.

4.5 Methodology

4.5.1 Cortisol detection using MIPs biosensor.

The following procedure includes the most common standard steps utilized to detect cortisol. First, the MIP cortisol sensor was connected to the LCR meter via its working electrode and Ag/AgCl reference electrode. Also, the LCR meter was logged to a laptop. Then, 500 µL of 0.1M phosphate buffer (pH7) was applied to the sensor to activate the sensor and measure the capacitance of the buffer after 24 hours. Finally, 500 µL of diluted cortisol was applied to the sensor and spiked to the maximum capacity in about 30 mins. Also, the sensors were tested for detection of cortisol within the physiological range (20-60-100-140) ng/ml.

4.5.2 MIP cleaning

Five hundred µL of 0.1 M phosphate buffer is applied to the textile surface to clean the biosensor. The Ag/AgCl reference and Auxiliary electrode are connected to the function generator to replicate CV devices runs. So, the generator supplied triangle wave potentials in the range of +0.9V to 0.9V at 0.1 V/s. In general, the washing process lasted twenty hours. Blank tests were run before and after the cleaning.

The exact process will be applied between each set of tests of the linearity of the cortisol detection within the physiological range. In addition, the first test after the cleaning process of each set was compared.

4.5.3 The resistance factor of the MIP

As a method for impedance spectroscopy, we found that resistance is our experiment's primary measurement parameter. Therefore, critical points of resistance must be considered in these testing. The initial objective is to determine the resistance range during the cortisol detection spike. In addition, the lowest resistance point has been obtained simultaneously with the maximum capacitance response.

During this test, Putty software captured the data from the LCR meter logging, counting the measured C and R parameters. First, the data will be extracted manually using an Excel spreadsheet, and then, the data will be analysed.

The analysis focuses on identifying the resistance behaviour and its relation to the capacitance.

4.5.4 Linearity tests

The initial biosensor sensitivity test involved exposing the sensor to four concentration levels, all of which fell within the physiological range. The concentrations measured were 20 ng/ml, 60 ng/ml, 100 ng/ml, and 140 ng/ml. Each experiment involved applying 500 µL of the dilution to the sensor's surface and observing its capacitance response. Additionally, resistance is monitored as resistance decreases with increasing concentrations.

4.5.5 Repeatability tests

To validate the repeatability of the detection results, the sensitivity test of the following concentrations is done twice, for a total of two tests per concentration: 20,

60, 100, and 140 ng/ml. First, a CV cleaning is performed 48 hours between each set of testing. The 48-hour interval between each concentration measurement ensures that the surface is dry.

4.5.6 Real-time monitoring of drops

This test investigated the device's short-term sensitivity to cortisol by observing the biosensor's transient response at various phases of resistance before and after the cortisol spike. After adding 60 µL of the mixture of buffer and sweat, the mixture is removed from the surface. Different 10 µL of cortisol concentrations, only buffer, and only sweat drops are examined. After each drop, the liquid is removed and try to test the drop effect at the same levels of capacitance and resistance.

4.5.7 Small drop test

This test is performed to determine the smallest amount of liquid capable of spiking the capacitance of the sensor to replicate the detection of sweat drops. A 100 ml drop of synthetic sweat has been applied to the sensor surface. In addition, 50000ng/ml of cortisol was added to the sensor interface for the second trial. Each test was conducted on a dried sensor.

4.5.8 High concentrations selectivity tests

Three concentrations have been diluted from the cortisol stock to check the sensor selectivity in high concentrations. The first dilution was 75000ng/ml, the second dilution was 50000 ng/ml, and the final one was 25000. 500 µL samples of each were applied on the sensor surface and waited 24 hours to observe the higher response possible.

4.5.9 CV run simulation test.

In this experiment, the working and reference electrodes of the biosensor will be connected to the LCR meter, while the counter electrode will be connected to the function generator. Then, 250µL of the phosphate buffer is introduced to the biosensor interface, and the capacitance is measured until stability is achieved. Following this, 20 µl of 75,000 ng/ml cortisol will be added. Finally, using an Excel spreadsheet, the test results were exported from the putty software and examined.

Chapter 5: Results

5.1 Cortisol detection

The MIP testing revealed the usefulness of molecular imprinting technology employing impedance spectroscopy techniques for ensuring sensitivity and detecting cortisol in sweat. Results from varied cortisol standard concentrations for MIP-based sensors are depicted in Figure 9.

The MIP demonstrates a superior sensitivity to cortisol due to receptor-specific cavities placed within the film. In addition, increasing cortisol concentrations increase the capacitance and decrease the resistance of the sensor interface. As a result, the sensor was able to detect physiological levels of cortisol in human perspiration.

5.2 Linearity test

The MIP succeeded in differentiating between different concentrations of cortisol, all within the physiological range of cortisol in human sweat. For 20ng/ml, the sensor has registered 166 nF in capacitance, 170 nF for 60 ng/ml, 175nF for 100 ng/ml and 176ng/ml for 140 ng/ml, as can be seen in figure 9.

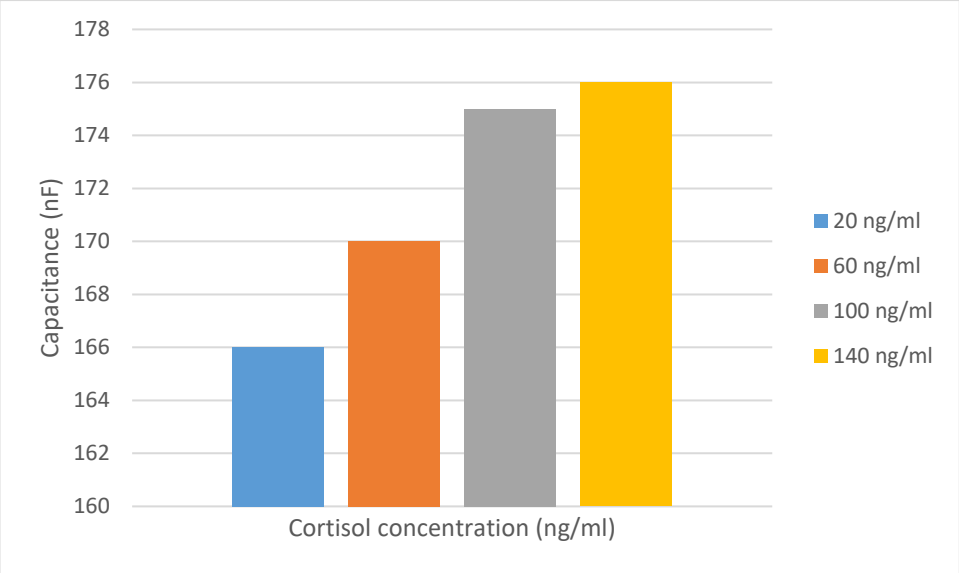


Figure 9

Capacitance response of MIPs biosensor to different cortisol concentrations within the physiological range.

5.3 Drops tests.

Drops of 10 µL of sweat and 50000ng/ml of cortisol will be dropped on the sensor's surface at a capacitance of 81 pF and 33KΩ resistance. The first two experiments show a higher cortisol response at 350pF while the similar drop of artificial sweat was raised to 290pF. However, after repeating the experiment for the third time, the reaction to the cortisol was poor compared to its reaction to sweat.

The experiment is repeated in lower resistance: 7.8 nF capacitance and 1.2 KΩ resistance. After removing the liquid from the surface, the capacitance was reduced to 6.8 nF. Then, adding only 10 µL of artificial sweat, the capacitance was raised from 6.8 nF to 7.2 nF. After that, the liquid was removed, and the capacitance was reduced to 6.8 nF. Once we added the cortisol drop, the capacitance was raised from 6.8 nF to 7.8 nF.

After that, at a capacitance of 7 nF, the experiment was repeated with 20 µL of cortisol and artificial sweat. Again, the sweat sample raised the capacitance from 7nF to 7.6 nF, while the cortisol sample increased it from 7 nF to 8.8 nF.

However, on the third attempt, both cortisol and sweat reacted the same, and both hit 8.8 nF. As seen in figure 10.

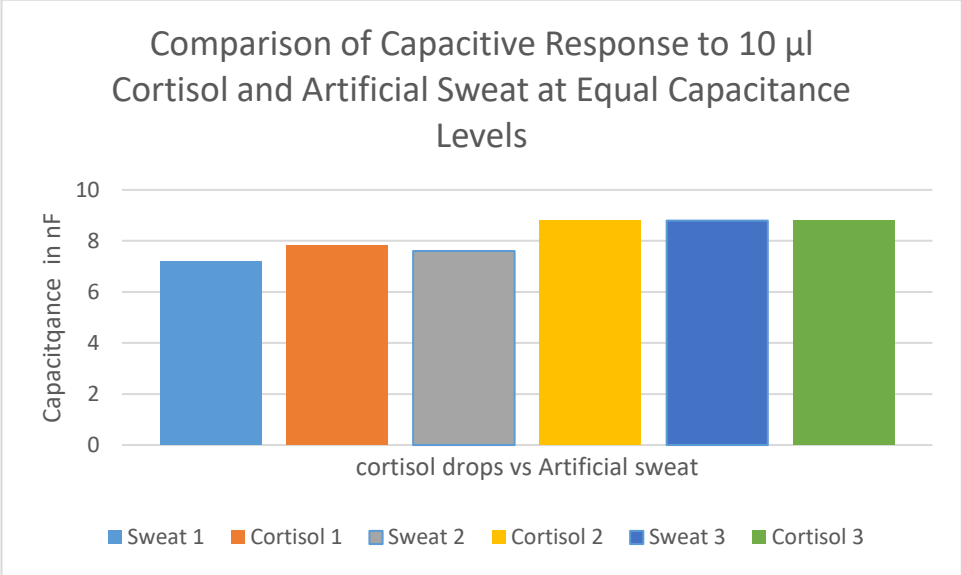


Figure 10 Capacitive response comparison between 10 µl of cortisol and artificial sweat at the same level of capacitance. The graph demonstrates the respective changes in capacitance values for both cortisol and artificial sweat samples.

5.4 One drop test on a dry sensor

100 µL of cortisol has shown a higher response than artificial sweat. The sensor responded to the cortisol with 1.21 nF at 4.5 KΩ resistance. On the other hand, artificial sweat recorded 0.86 nF at 6.38 KΩ resistance, as seen in figure 11.

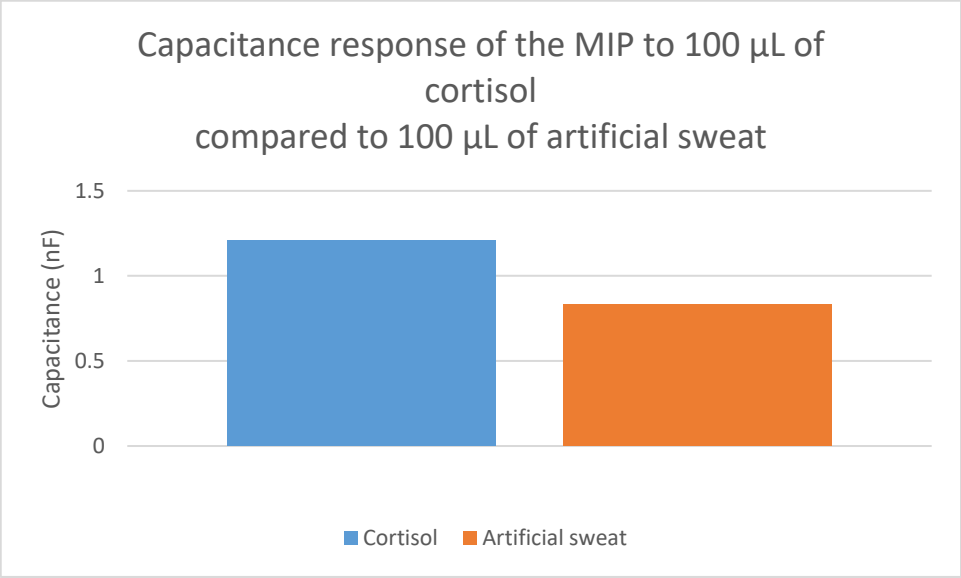


Figure 11

Shows capacitance response of the MIP to 100 µL of cortisol compared to 100 µL of artificial sweat.

5.5 High concentrations selectivity test

The sensor has detected different ranges of cortisol concentrations. For example, it registered 0.988 nF for 25000 ng/mL, 3.1 nF for 50000 ng/mL, and 3.8 nF for 75000 ng/mL, as shown in figure 12.

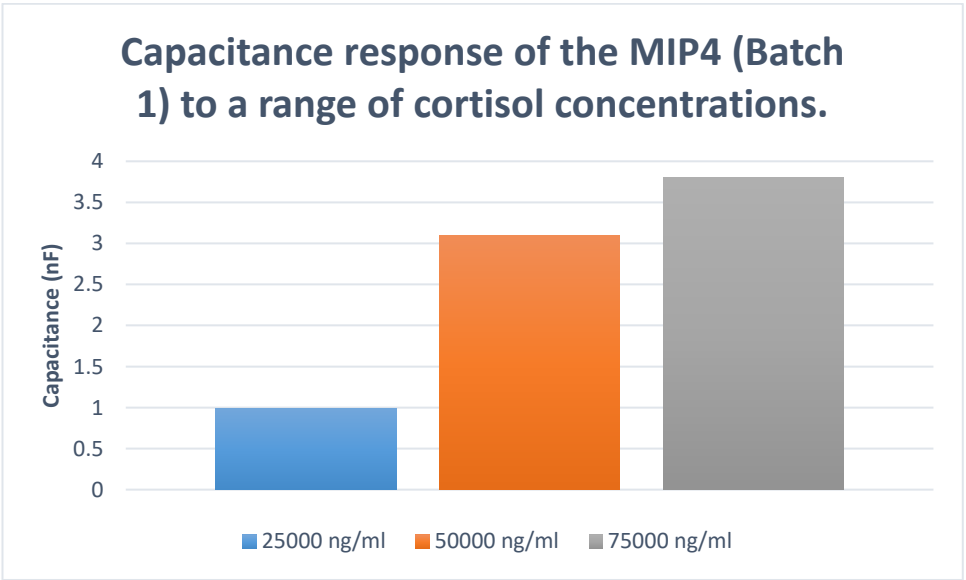


Figure 12 Shows the difference response of the MIP4 (Batch 1) to different range of cortisol concentrations.

The results presented in figures 9, 10, 11 do indeed demonstrate that the designed MIP sensor detects cortisol.

5.6 Cleaning results

After detecting cortisol, the sensor's ability to clean must be confirmed to ensure its reusability. Following a series of cortisol tests, 500 µL of phosphate buffer was supplied to the sensor. The sensor's reaction to a contaminated buffer was 3.08 nF. However, after cleaning the sensor surface, the same quantity of buffer was applied, and the reading was 0.949 nF. Due to the number of ions held in the dielectric area, the results indicate a higher reaction before cleaning. The reduced reaction suggested that the accumulated ions had been removed after cleaning, as seen in figure 13.

Also, comparing figure 14 (Linearity tests after cleaning) with figure 9 (unwashed) reveals that the cleaning process eliminated the cortisol. A linear detection was observed in the lower ranges.

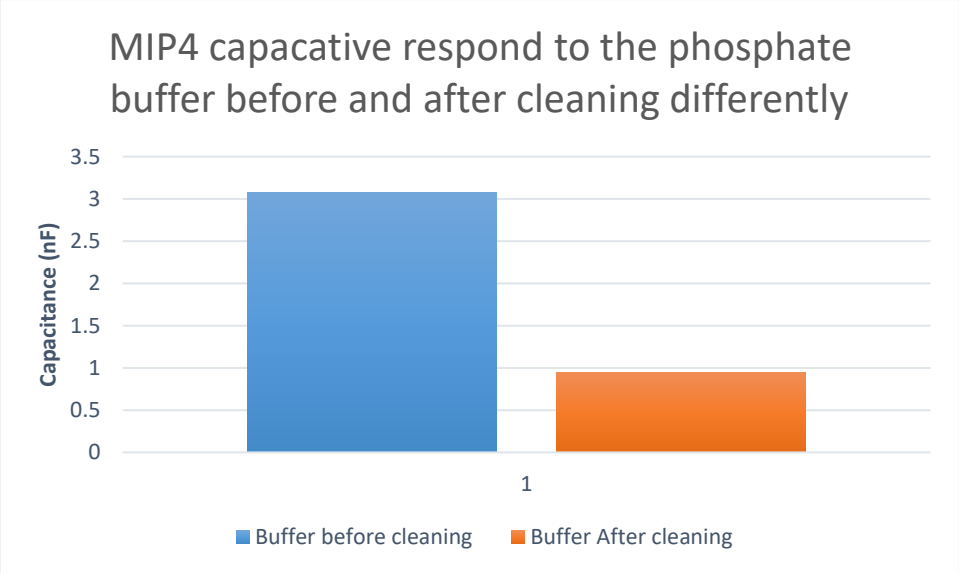


Figure 13 shows MIP4 respond to buffer before and after cleaning differently.

5.7 Repeatability tests results after cleaning.

The results were 20ng/ml for the first set of tests. The sensor has registered (166, 170, 175, 176) nF for (20,60,100,140) ng/mL, respectively. While the second set recorded (38.5, 41.1, 53.5, and 180) nF for (20,60,100,140) ng/mL, respectively. As can be seen in figure 14.

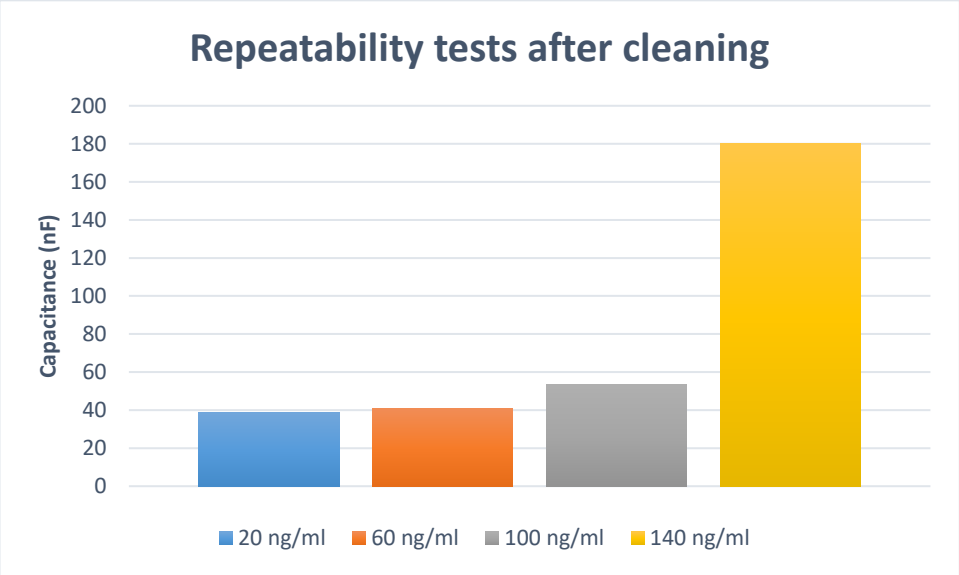


Figure 14

Presents the second set results of the repeatability tests different range of cortisol concentrations.

However, As shown in Figure 15, prolonged use of the same sensor can result in the build-up of mineral salts on the sensing surface, which may affect the accuracy of the measurements.

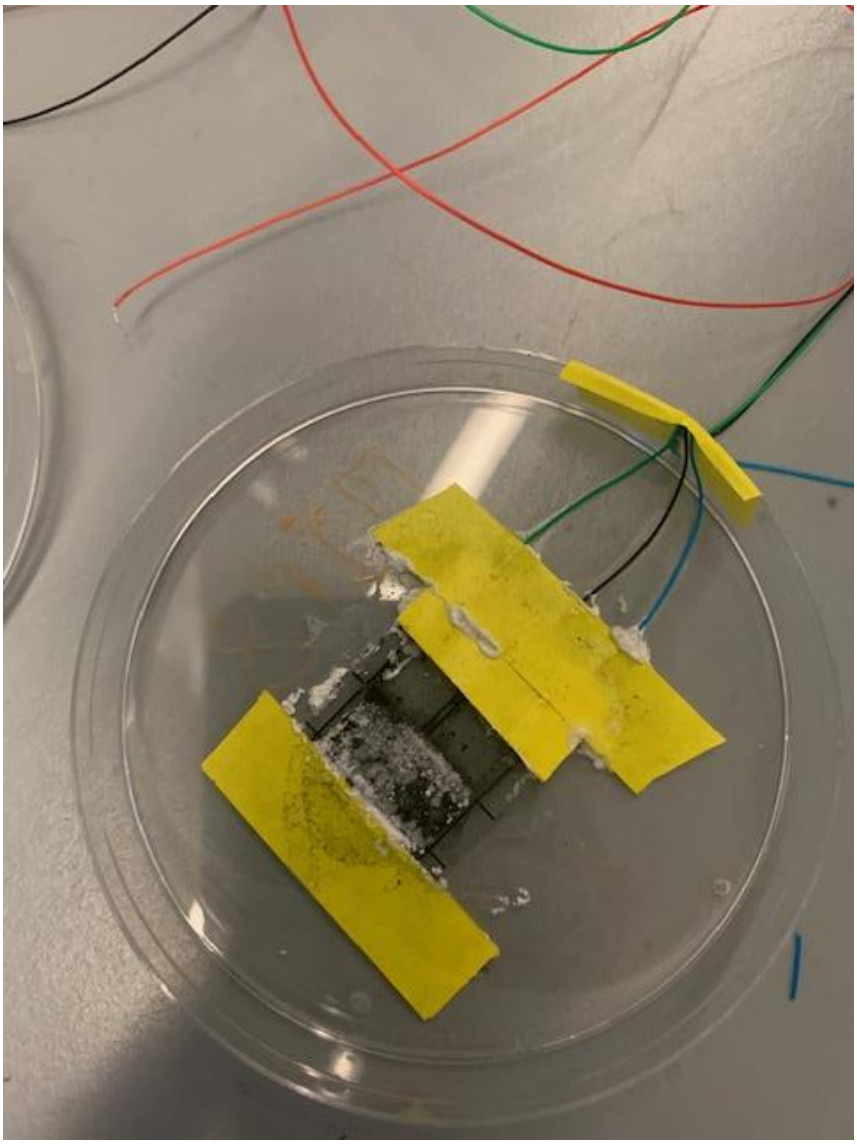


Figure 15 Shows damaging of the electrode's surfaces and salt accumulation on the sensing surface after using the sensor about 50 times.

5.8 Resistance tests

In an activated biosensor, the resistance is varied between each sensor depending on the time of the last use of the sensor and how clean it was. However, once the sample was added to the surface, the resistance droped, and the capacitance increased gradually. For example, in a biosensor from the second batch, the dried biosensor had a capacitance of 14 pF in 8 MΩ resistance. When the resistance is 18 KΩ, the capacitance is 66 pF. As can be seen in figure 16.

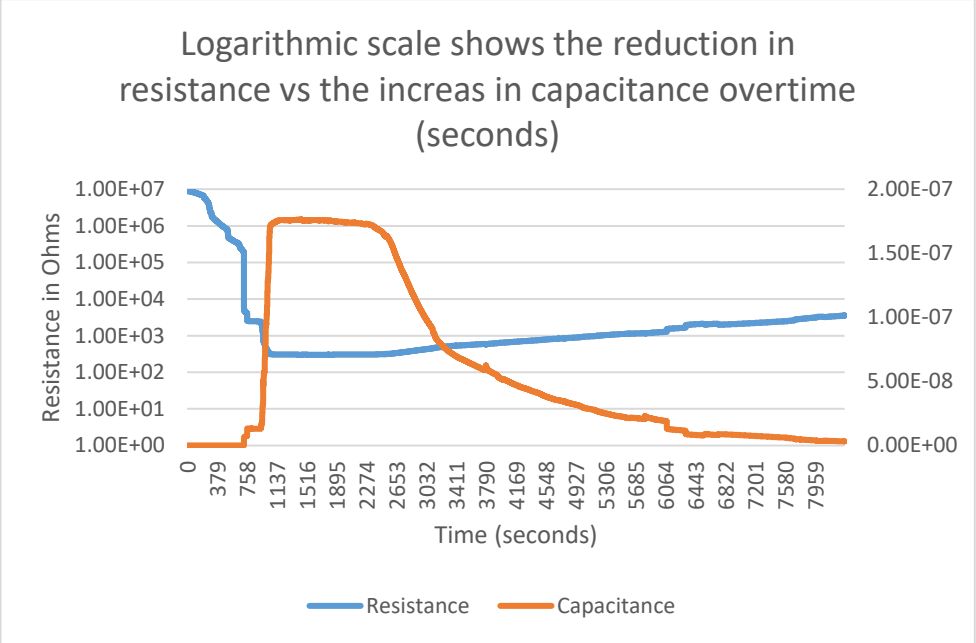


Figure 16

A) Shows the resistance change in the detection of 140 ng/ml diluted cortisol sample. B) shows change of the capacitance of the sensor in the same experiment.

Also, it has been noticed that the spike was 81 pF to 6 nF when a sudden drop in resistance between 9 KΩ to 1 KΩ in the 140ng/mL sample, as seen in table 3.

In a test of cortisol using a biosensor from the second batch, after adding 500 µL of 20 ng/mL of cortisol, the capacitance was spiked from 45.9 pF to 11.8 nF when a sudden decrease in resistance from 470 KΩ to 3.81 KΩ as it can be seen in table 4.

Sample	**Sample number (3 samples per second)**	**Capacitance**	**Resistance**
140ng/ml	2156	8.15E-11	9.49E+04
140ng/ml	2157	6.05E-10	2.24E+04
140ng/ml	2158	1.23E-09	1.63E+04

Table 3

Shows the change of capacitance and resistance at the beginning of the cortisol spike when 500 µL of 140 ng/ml of cortisol is tested.

Sample	**Sample number (3 samples per second)**	**Capacitance in Farads**	**Resistance in Ohm**
20ng/ml of cortisol	1956	4.59E-11	4.72E+05
20ng/ml of cortisol	1957	3.53E-10	6.10E+04
20ng/ml of cortisol	1958	5.08E-09	6.51E+03
20ng/ml of cortisol	1959	1.18E-08	3.81E+03

Table 4 Shows the change of capacitance and resistance at the beginning of the cortisol spike when 500 µL of 20ng/ml of cortisol is tested.

Before the sudden resistance change, the capacitor acts as an open circuit with high resistance and blocks the current. After the spike, with the resistance reduction, the capacitor starts to function. Thus, the sensor's response becomes more accurate once the changes happen. Based on the results and the physical monitoring, the ideal resistance range for the current biosensor design is between 2KΩ and 300 Ω.

Moreover, when 20ng/mL was tested, it recorded 320Ω as the lowest resistance point. Moreover, it is 309Ω for the 60 ng/mL test, 305Ω for 100 ng/mL. Finally, 302 Ω for 140 ng/mL test, as can be seen in figure 17.

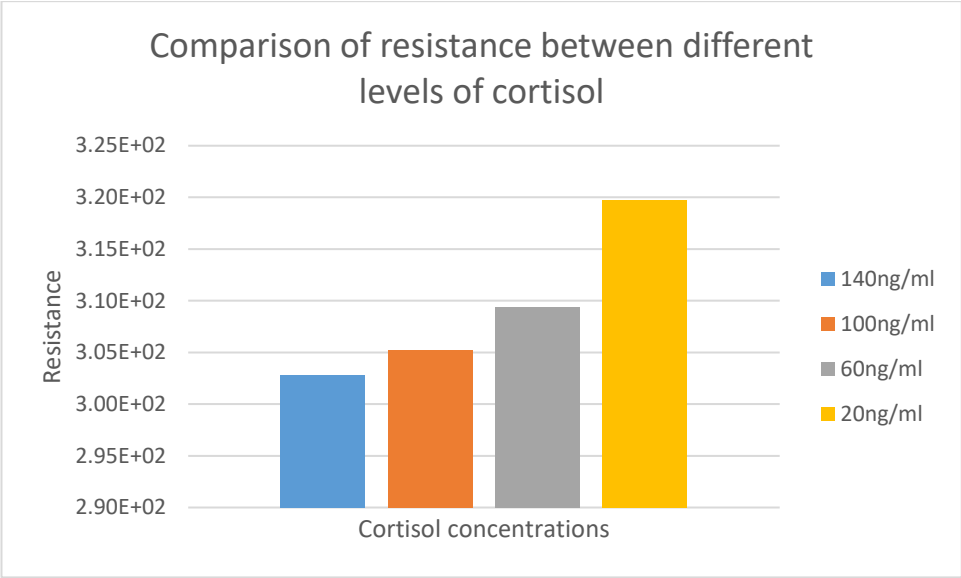


Figure 17 Compares the resistance of various levels of cortisol concentration**.**

The cortisol concentration in the sample may alter the recorded resistance when measuring diluted cortisol using an LCR meter. This is since the concentration of cortisol in the buffer determines the conductivity of the mixture. Highly concentrated cortisol has higher conductivities and lower resistances than diluted cortisol. The results demonstrate linearity among the four samples with varying concentrations.

5.9 CV simulations test

In this experiment, the Simulation recognized the decrease in capacitance by real-time monitoring. In each of the three repetitions of the experiment, the reduction has been observed. The baseline buffer capacitance was approximately 43 nF. Figure 18 demonstrates that the capacitance was lowered to 35 nF with the addition of cortisol. After that, the capacitance began to climb until it reached 40 nF; another cortisol sample was added, and a dip to 30 nF was seen. In the final test, once the capacitance has been increased to around 34 nF, cortisol is added to the interface, decreasing capacitance to 29 nF.

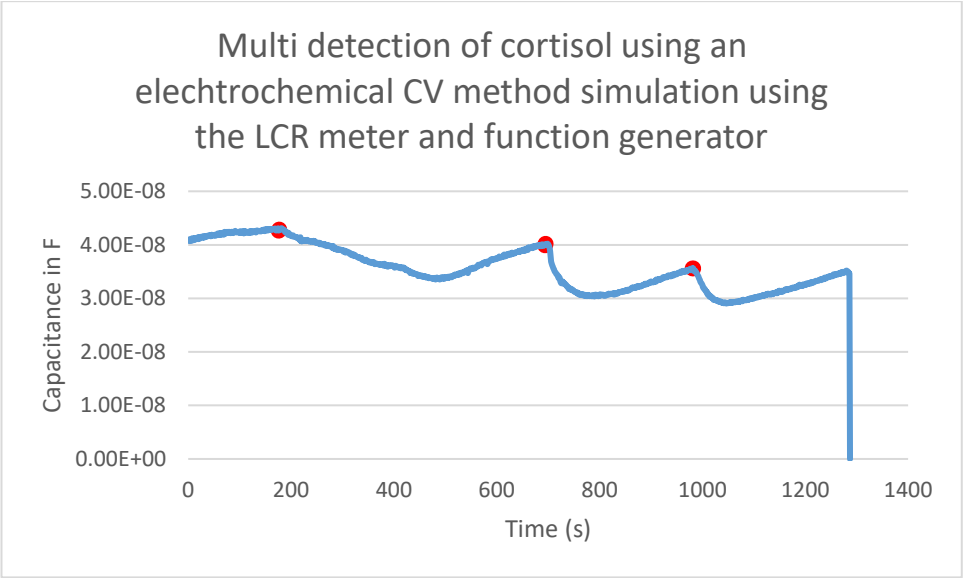


Figure 18

Describes three detections of cortisol in row in real time monitoring using the LCR meter and function generator each peak is in red after each peak the drop of cortisol was added.

Chapter 6: Discussion

6.1 Cortisol detection

Electrochemical impedance spectroscopy has proven to be a valuable tool for physiological cortisol detection. This is because the capacitive MIPs biosensors respond higher to greater concentrations for each test and through the different batches of fabricated MIPs.

6.2 Drops tests during various stages of resistance.

As evidenced by the data, as seen in figure 10 the sensor's sensitivity is ineffective prior to the capacitance, and resistances spike positively and negatively, respectfully, as there are no significant variations between the artificial sweat and cortisol readings. In contrast, the differences between cortisol and sweat became apparent after the capacitance spike accumulated in low resistance reading.

6.3 One Drop tests from a dry sensor

100 µL of cortisol produced a sensor response of 1.21 nF and 4.3 KΩ resistance, but 100 µL of sweat produced only 830pF and 6.38 KΩ resistance. However, it is difficult for the sensor to detect cortisol before the spike. It must be sufficiently fluid (less than 10 KΩ resistance) to differentiate between cortisol-containing and cortisol-free solutions. As seen in the results, the difference is low between both responses, and it might be varied based on the initial resistance of the sensor.

6.4 Linearity tests in the physiological range.

As can be seen in figure 9, the biosensor can distinguish between all four samples within different concentrations. The first three samples have more linearity and close results compared to 140ng/ml of cortisol concentration. Even though the second set of tests has a different range of responses, the first three tests have a more gradual increasing line followed by a sharp response. This pattern is repeated twice.

As the detection signal increases in capacitance with the increase of the cortisol concentration, several factors can affect the practicality of the sensor. Even though the current design of the sensor can confirm that the range of cortisol concentration is within the physiological range diluted in buffer, the direct detection of natural drops of sweat on the skin has yet to be investigated. Even though the LCR meter is not designed to measure the concentration of the substance directly, it indirectly

succeeded in measuring the cortisol concentrations as different concentrations present different electrical properties. However, a wearable device might have a different result based on the meter's sensitivity.

By using impedance spectroscopy to quantify the change in the electrical characteristics of the MIP biosensor as a function of the target molecule concentration, MIP biosensors may be employed for accumulative measurement. For example, low-concentration target analyte measurements taken after a high-concentration test may produce an unexpectedly positive result. It is possible that the MIP biosensor's capacitance was impacted by ions left over from the high-concentration test that remained adsorbed on its surface.

The binding sites on the MIPs may become saturated with ions when subjected to a high target analyte concentration, leaving the biosensor inefficient in binding any further ions. In addition, due to the presence of the remaining ions from the high-concentration test, the biosensor may have a more significant reaction to a lower-concentration sample. Notably, the application of MIP biosensors for accumulative measurement employing impedance spectroscopy methods will depend on the individual properties of the MIP biosensor and the standardized measurement conditions. In addition, the sensitivity and reliability of the measurement will rely on the design of the MIP biosensor and the test conditions.

6.5 High concentrations test

The sensor differentiated between three samples with high concentrations, as seen in figure 12. All sample responses were lower than the sensor response to cortisol within the physiological range. The saturation effect is the situation that arises due to the high binding capacity of MIPs. Saturation of the MIPs with the analyte, when there are no binding sites accessible, reduces the biosensor's responsiveness at very high concentrations. Saturation can happen at various concentrations depending on the biosensor's setup. Additionally, the saturation effect can be created by overloading the sensor with high analyte concentrations. This can cause some MIPs to dissociate from the surface, limiting the total number of MIPs available to bind the analyte. Furthermore, an overload of analytes can reduce biosensor responsiveness because different analyte molecules compete for the same binding sites. The sensitivity of the sensor to low concentrations is high. This may be explained by the theory of

impedance spectroscopy, which states that higher concentrations correspond to greater capacitance. Thus, the outcome of this test could reveal the highest detectable concentration limit.

6.6 Repeatability

In repeatability testing, the biosensor demonstrated that it could detect various cortisol concentrations. Nevertheless, it is not easy to maintain the same level of accuracy between each detection and cleaning. Numerous elements play a crucial part in this incident. First, a capacitive biosensor is an accumulative indication since it represents the number of stored charges. Adding additional drops of cortisol will increase capacitance and decrease resistance. In this scenario, the sensor can only detect more significant concentrations after each detection; decreasing the sample concentration will result in erroneous readings.

Secondly, the number of stored charges may also be affected by the cleaning procedure's duration and quality. Therefore, repeating the same experiment may result in successful detection but at a different capacitance level.

Thirdly, using a relatively large quantity of liquids can result in the loss of surface materials from electrode surfaces. Consequently, the damaged electrodes respond less to cortisol than they did in their original state, as seen in figure 15.

Fourth, utilizing the same sensor for extended periods might result in the formation of mineral salts on the sensing surface, which can compromise the accuracy of the reading, as shown in figure 15.

Finally, room temperature may also be a factor, as temperature influences the resistance of liquids (Murdock *et al.*, 2012).

6.7 Resistance

The resistance of the sensor plays a crucial role in our experiment. Dried sensors need a significant amount of liquid in measuring levels (which is lower than approximately 5 KΩ). The brand-new MIPs biosensor has a resistance of more than 100 MΩ. To reduce it, it needs about 500 µL of buffer or liquids and adding drops on the electrode's surfaces. The hydrophobicity of the sensor's surface also is a crucial factor

that might affect the resistance response to the added liquid. After the activation of the sensor by reducing the resistance, experiments can be run efficiently. However, after running the experiments, maintaining the dryness of the biosensor at the same level of resistance is difficult. The resistance at this stage is based on the ions remaining within the textile and a physically unremovable liquid. For example, after a day of running an experiment of 500 µL of cortisol diluted in buffer, the resistance usually is less than 10 MΩ depending on the number of experiments done before and how clean the sensor is. Having lower resistance at the beginning of a test will increase the response number of the capacitance while having higher resistance will result in a lower response. Therefore, it might be ideal to start a set of tests at the same resistance range as much as possible. Furthermore, it might be ideal to have the same time difference between each test so that the resistance would be at the same range before each test. To prevent the need of buffer Zhao et al. (2022) introduced a passive continuous analysis patch to monitor cortisol levels at normal levels. Microfluidic channels were integrated into the patch to achieve this. Sweat is extracted from the gland through the microfluidic channel and transported to the sensing surface. Approximately 40 minutes were required for the sensor to be immersed in sweat. As the technique targets the sweat at rest and stimulates the sweat glands to produce sweat, it is beneficial for events that are not stressful. On the other hand, emotional sweat with higher cortisol levels is a natural reaction. The development of microchannels for sweat collection may have merit but without the addition of stimulation techniques, such as iontophoresisAdditionally, Cortiwatch used a buffer to wash the device and determine the baseline resistance before running the test. A cartridge-style system was used to eliminate the need for a massive buffer on the skin, which allowed the sensor to be interfaced with the skin and isolated from the environment outside. By using this system, sweat could be collected directly from the subject, eliminating the need for excessive buffer application. During the process, the patch was applied to the cleaned surface of the participant's wrist to collect data. In order to ensure the subject's convenience and a non-invasive monitoring experience, CortiWatch was connected to a computer for data collection and analysis. The device successfully detected cortisol in 3 µL of sweat. (Rice *et al.*, 2019). Storer et al. (2018) used a nitrogen gun and deionised water to clean the surface after each experiment. This was to maintain the same conditions of the sensor after exposure to an analyte.

Since they maintain the same capacitance level, it may be argued that resistance is reset since it increases while capacitance decreases. This was done to provide the same baseline data for each experiment. This process allowed the researchers to accurately compare the results of each experiment and measure the impact of the analyte on the sensor. By modifying the sensor design, such as integrating microchannels, or using physical solutions, such as adding the buffer to activate the device and removing it, researchers were able to prevent the need for large buffer volumes. The unremovable buffer determines the baseline of the system's resistance in this scenario.

The spike of resistance is a factor to be considered. After adding the sample to the surface and the resistance starts to be reduced, it will reach a point when the resistance would significantly reduce in parallel with the increase of the capacitance. This point is varied between both batched received biosensors. For example, in our first batch of biosensors, the capacitance is typically spiked to 1 nF when the resistance reading is 5 KΩ. In comparison, it is 5 nF when about 5 KΩ in the second batch of sensors. The detection range starts lower than 8 KΩ when the dramatic negative relation establishes between the resistance and the capacitance. These negative relations can be observed in the resistance range lower than 1 kΩ to 400 Ω. Within this capacitance range, the capacitance spike is remarkable as it rises sharply from 1 nF to the samples' maximum capacitance.

In comparison, the change in capacitance is slower before the spike, as shown in figure 16. This is because, before the spike, the resistance of any liquid decreases similarly as the system's liquidity rises. However, the lowest capacitance following the peak varies according to the cortisol concentrations of the samples. This may limit the applicability of the existing MIPs device, as it requires at least 500 µL of samples to reach the spike faster. Therefore, it can be advised that the system's size be decreased to lower the amount of liquid necessary to spike. Alternately, altering the textile's characteristics can make it more absorbent, have fewer liquids needed to saturate with, or be less hydrophobic on the surface. Also, as our utilized biosensor was created for the CV electrochemical approach, it may be worthwhile to adapt it for electrochemical impedance spectroscopy, as it necessitates a working electrode ten times the size of the counter electrode (Lisdat and Schäfer, 2008).

In a conceptual wearable sweat cortisol device, resistance can also indicate sweating. In terms of power saving, the capacitance reader starts reading once a drop of resistance starts. Due to the absolute humidity caused by the water content on the sweat biosensors, resistance can signalize the sweat loss level (Taylor and Machado-Moreira, 2013). The overall resistance and capacitance drop and rise when sweat flows down the microchannel. In a hypothetical design for wearable technology, resistance might serve as a signal of perspiration, therefore activating capacitive measuring. Additionally, it may assess sweat concentration, as higher concentrations result in a lower value of resistance using wearable sweat loss measuring devices (SLMDs) (Zhong *et al.*, 2022).

As seen in Figures 14a and 14b, the resistance reading decreases significantly at the beginning of the experiment. The logarithmic scale decreased from 10 MΩ to approximately 1 KΩ. In contrast, there is a slight rise in capacitance synchronously as it grows from 18pF to around 1 nF within the same given period. The drop in resistance from 1 KΩ to about 400 Ω boosts the sample's capacitance to 176 nF.

To summarize, it is preferable to have a low resistance to measure the capacitance response correctly. This is because the resistance of the device being measured might impact the accuracy of the capacitance measurement.

For instance, if the device's resistance is considerable, it may result in a significant voltage drop across the device, which can alter the capacitance measurement. This is especially critical for measuring exceedingly small capacitances, as the voltage drop across the device might represent a substantial portion of the overall voltage applied.

Conversely, if the device's resistance is low, it will limit the capacitance measurement. This will result in a more reliable capacitance measurement.

6.8 Cleaning test

There is a clear distinction between the tests of buffer samples using phosphate buffer on a sensor that has been cleaned and buffer on a sensor that has not been cleaned. The response to a sample that has been cleaned is significantly reduced, which may suggest that the ions have been removed from the dielectric layer. On the other hand, the high response of a contaminated buffer could be attributable to the ions stored in the dielectric layer.

As shown in figure 9, 20 ng/mL of cortisol was measured using an uncleaned biosensor. It revealed 165 nF. However, once the linearity tests were complete, the sensor was cleaned as previously described. After testing 20 ng/mL of cortisol, the second series of linearity tests are initiated, as is seen in figure 14. The data indicate that the sensor only responded to 38.5nF. These data are evidence that the cleaning hypothesis has been answered.

There has also been evidence of a reduction in signal after cleaning in several studies. Parlak et al., (2018) tested a range of concentrations to determine the device's response, with measurements conducted at low, medium, and high concentrations. Following the measurement, the sensor surface was regenerated by rinsing with a washing solution and sonicating for 30 seconds. In both increasing and decreasing concentrations of cortisol, the device demonstrated a stable response. However, in order to understand how the device responds when saturated with cortisol, the cortisol concentration was increased tenfold (up to 5 mM) and then gradually decreased from 5 mM to 0.001 µM. When the device was saturated, there was no response to decreasing cortisol concentrations. In these experiments, saturation of the device and molecular binding were demonstrated to be essential factors in the sensing process. Washing and sonicating the sensor surface may remove contaminants or residual substances affecting the sensor's dielectric properties. Consequently, this could affect the sensing performance and the response to cortisol levels. Additional factors, such as changes in surface chemistry, electrode properties, or binding interactions, may also influence the observed response. The exact role of the dielectric area in the observed changes would require further investigation. Kong et al. (2023) optimized the number of polymerization cycles, with 15 cycles found to improve the EIS response. However, increasing the cycles beyond 15 led to a decrease in the response, indicating the importance of finding the optimal balance. Furthermore, an experiment was conducted to develop MIP-silica hybrid particles to detect amlodipine. In order to remove the template (amlodipine), the particles were washed with methanol, and the absorbance of the supernatant was evaluated using ultraviolet visible UV/VIS spectroscopy. Upon successive washings, there was a decrease in absorbance, which indicated that the template had been removed. Four washes were required to remove the template altogether, leaving behind structurally adapted cavities or interaction sites within the MIP-silica hybrids.

In order to determine whether hybrid particles could rebind amlodipine, they were thoroughly washed and dried. In response to a standard amlodipine solution, the absorbance of the solution decreased, suggesting that the amlodipine molecules were taken up by the MIP-silica hybrid particles and adsorb to them. Consequently, the amlodipine concentration in the solution decreased. A measurable decrease in amlodipine concentration in the solution was observed due to the successful removal of the template from MIP-silica hybrid particles (Roshan et al., 2019). Kidakova et al. (2019) study investigated the possibility of regenerating and reusing protein molecularly imprinted polymer (MIP)-based sensors. The MIP sensor was regenerated using acidic and alkaline solutions to disrupt hydrogen and electrostatic bonds between the protein and the MIP surface. After the first regeneration cycle, the sensor's response significantly decreased. Also, another study involved injecting a regeneration buffer composed of MeOH and a buffer solution to regenerate the working electrode. Capacitance was measured using the current step method, in which a constant current was alternately applied to the electrode surface, and the resulting potential profile was used to calculate capacitance. The binding of the target analyte to the immobilized MIPs on the electrode surface decreased the capacitance registered. The Deviation values were below 4.2% after cleaning (Lenain et al., 2016). Roushani et al. (2022) needed to clean their MIPs sensor five cycles to restore its signal. A study by Amaly et al. (2021) found that MIPs nanospheres in water treatment are restored to 85% after seven cycles. However, cleaning the sensor for the eighth time resulted in just 48% of the cavities being regenerated. The process of cleaning may be adversely affected by over-cleaning.

A function generator can generate the waveform for cyclic voltammetry investigations. The electrochemical technique of cyclic voltammetry involves applying a voltage to an electrode submerged in a solution and measuring the current that flows through it. The current as a function of potential is plotted to create a cyclic voltammogram, where the potential is ramped up and down. Ones can use a function generator to create a ramp, square wave, triangle wave, or any other waveform for the applied potential.

Thus, CV is proven to clean a MIP biosensor. In cyclic voltammetry, the cell's potential is cycled from a low value to a high value and back down to a low value. This can be

utilized to eliminate undesirable impurities or other species that may be present on the MIP biosensor's surface. More importantly, it can be utilized to regenerate the sensor by removing the target analyte from the MIP material's surface and restoring the material's original structure.

The biosensor must be connected to an electrochemical measuring system to clean a MIP biosensor using cyclic voltammetry. The system would apply a series of voltage cycles to the biosensor using an appropriate electrolyte solution as the solvent (such as phosphate buffer).

In a wearable device to make a CV run using the internal circuit of a wearable device, it must use a built-in or attached calibrated function generator. Then, the MIP biosensor must be placed in an appropriate solvent, phosphate buffer. After that, the function generator applies a voltage ramp to the electrode. The voltage should be periodically ramped up and down within a specific range and utilize the device's internal circuit to measure the resulting current. The procedure must be repeated multiple times to ن selectivity. Of course, such cleaning process can be implemented in the future by the smartwatch itself without the need for the function generator.

Increasing the solution temperature can generally accelerate chemical reactions, including those that occur while washing the MIP biosensor. At higher temperatures, this can result in quicker biosensor cleaning. However, it is essential to note that the best temperature for washing a MIP biosensor will depend on the biosensor's design and the target that must be eliminated. Further research is suggested to determine the optimum temperature at which the cortisol can be effectively removed from the biosensor. Also, a specific range of temperatures might be affordable while charging the battery within the device part, which might be considered in future work to accelerate the cleaning process. For example, at different temperatures, Wei et al., (2018) discussed the effect of self-cleaning on the performance of the bovine serum albumin temperature-sensitive MIPs on a glassy carbon electrode , BSA-TMIPs/GCE biosensor. A redox probe is used to determine whether self-cleaning is efficient at different temperatures.

Results suggest that the BSA-TMIPs/GCE biosensor was significantly more sensitive to a 37 °C PBS buffer solution than to different treatment temperatures such as 20 °C, 40

°C, and 50 °C. In TMIPs/GCE, the temperature is observed to influence the structural changes of the biosensor contribute to its self-cleaning capability.

As a result of the polymerization, the BSA-TMIPs/GCE retain their original polymerization structure at 20 °C, as well as their interaction between monomers and templates. At 37°C, the lower critical solution temperature (LCST) is reached, and the poly(*N*-isopropylacrylamide) PNiPAAm chains undergo a conformational conversion, transitioning from hydrophilic to hydrophobic states. Through this process, the hydrogen bonding interactions between the templates and monomers are weakened, which facilitates the removal of BSA from the biosensor. However, it is possible that the structural conversion might not take place entirely at temperatures higher or lower than the LCST, resulting in residual BSA.

Moreover, applying potential cycling over a broader range (-0.8 V to +0.8 V) enhances the peak current ΔI response. By expanding the potential range, multiple-point electrostatic interactions are more rapidly destroyed, thereby assisting in removing BSA. Self-cleaning conditions are optimized by considering the effects of oxygen and hydrogen evolution processes. The optimized self-cleaning conditions for the BSA-TMIPs/GCE biosensor involve a temperature of 37 °C, potential cycling from -0.8 V to +0.8 V for 14 cycles, and a scan rate of 100 mV s.

Wei et al. (2022) examined the effects of temperature on BSA rebinding. According to the results, the binding capacities of the dual-stimuli-responsive thermo-responsive and electric-field dual-stimuli-responsive on a glassy carbon electrode, DR-MIHs/GCE biosensor were affected by temperature. The DR-MIHs/GCE biosensors effectively adsorbed and desorbed BSA by alternating between applied and withdrawn voltages between 20°C and 50°C. Upon heating to 20°C, the imprinted sites, conformational sizes, and functional orientations of the imprinted membrane closely resembled those formed during the polymerization process, which increased the ΔI of the redox probe. As a result, the target protein was successfully complemented. The application of a specific voltage at 37°C caused a decrease in the flux of soluble BSA, indicating that it was released into solution after the application of the voltage. The subsequent increase in ΔI at 20°C indicated the recombination of BSA into the DR-MIHs/GCE biosensor. These findings highlight the importance of temperature control during the

rebinding process, as it directly influences the affinity and stability of the biosensor for the target.

6.9 CV run simulation.

This experiment is essential to the concept of a practical wearable device for real-time cortisol monitoring. Most studies use an external CV device to perform the runs in the literature. The data is extracted through a voltammogram, and then the capacitance is calculated using scientific formals. In contrast, our method is more practical at the wearables level. As it can monitor real-time capacitance, a sudden drop can be noticed immediately. This simulation can be afforded in wearable devices using an internal capacitive reader and specified waveforms and their shapes, and the required frequencies using a built-in function generator in the wearable device.

Compared to the CV simulation methods and spectroscopy, CV simulations are more accurate as they can be seen in real-time monitoring. Another advantage is that the resistance factor has fewer impacts on detection accuracy, unlike impedance spectroscopy, where it is crucial. On the other hand, continuous monitoring using a CV run is energy-consuming as the voltage needs to be applied constantly. They can be combined in a wearable device to take advantage of both technologies. The LCR meter was connected to the working electrode and the reference electrode in our experiments. Thus, in a conceptual device, both modes apply as the capacity reader is always connected to the sensor in spectroscopy mode, which can be used consistently with minimum energy requirements. Urgent cortisol tests can be done by activating the CV mode, the counter electrode is connected to the function generators.

In the first mode, a smartwatch would indeed be designed to detect cortisol in perspiration using impedance spectroscopy. The MIP biosensor would be placed inside the smartwatch, which would absorb the sweat. The impedance spectroscopy would be performed to detect variations in the resistance and capacitance of the biosensor, which would signal the presence and intensity of cortisol in the sweat; however, it could take longer to achieve the full response. It might be useful in monitoring day to day cortisol level.

In the second mode, the smartwatch would detect cortisol in sweat using cyclic voltammetry (CV). CV is a technique that use a potential scan to detect variations in the current flowing through the biosensor, and it is effective for detecting variations in

the oxidation and reduction processes of the target analyte. The CV would be accomplished by providing a voltage to the counter electrode and monitoring the biosensor's current flow. This mode has the advantage of providing rapid detection and can be used for real-time monitoring, but it is battery consuming.

MIPs also can be regenerated during the charging using CV runs. But it is worth knowing that the voltage applied during the detection has different values and can be adjusted while manufacturing.

Chapter 7: Conclusion

7.1 Conclusion

The evaluation of stress biomarkers revealed that stress detection is an emotional event. Included in future investigations were possible biomarkers. This article examines electrochemical stress biomarkers and concludes that cortisol is the most significant stress biomarker since it is observable in sweat. In addition, this review examined alternative stress biomarkers in perspiration, such as cortisol metabolites and antistress chemicals. Most studies in this sector have established cortisol detection methods.

Based on a literature review of the current research on the electrochemical detection of sweat cortisol, it is advised that an electrochemical impedance spectroscopy method be applied to a molecularly imprinted polymer capacitive biosensor. The possible cause for the delay in the widespread distribution of sweat cortisol wearable devices is the need for technologies to accommodate the commercial viability of wearable devices that detect sweat biomarkers. A conceptual detection strategy based on improving the practicability of capacitive detection on a wearable device was provided to prove the concept.

In addition, a washable MIP in a wearable device was an unresolved issue, as most present MIP biosensors are disposable. According to the literature, cortisol molecules are extracted from the MIPs sensor utilizing the electrochemical CV approach during manufacture. This step prompted the current work to remove cortisol utilizing the exact voltage parameters but in a novel manner by employing a functional generator instead of CV devices. However, these signals can be utilized in wearables without needing a large CV device attached.

The motivation for the current research came from the rise of the mental problem society in terms of the difficulty of emotional communication with people suffering from emotional expression due to mental health problems such as autism. Therefore, the goal of a wearable device that can alert wearers or their carers to ease communication or prevent a harmful event was the goal of the current research.

A published review paper in an academic journal identified that cortisol is the primary stress biomarker. Also, the review suggested that MIPs biosensors are the most

advanced method to detect it. Based on the result, further studies have been conducted to prove the concept.

7.2 Challenges

Even though the electrochemical impedance spectroscopy method has proven to be more applicable for wearable devices as the direct measurement of capacitance and resistance is much easier to be employed in wearable systems compared to the CV method, which needs external tools to apply CV run, there are several limitations to be addressed in the future. First, the detection range needs low resistance, which requires a vast amount of uncomfortable liquid. In this scenario, the calculation of the cortisol concentration must be addressed as the added liquid dilutes the actual concentration. Alternately, future research could examine reducing the resistivity of a manufactured sensing surface by adding more low-resistivity elements such as carbon nanotubes, gold nanoparticles, graphene, and/or metal nanoparticles based on the specific scientific requirements.

Consider the amount of liquid needed for accurate capacitance measurement. Thus, the effect of the liquid's variability on the measurement is reduced. To get the most accurate readings possible, it is essential to ensure the liquid is homogeneous and thoroughly mixed. Another technique to accomplish CV using a solid medium is employing an electrode modified with an electroconductive polymer that can immobilize recognition molecules. This can improve analyte detection and eliminate the need for a buffer. However, there may be limits to executing CV with a solid medium compared to buffer-based CV. For instance, the electrochemical reaction of the analyte may vary in a solid media, which can impact the sensitivity and reproducibility of the results. In addition, solid-state sensing materials may have a limited lifetime, which may decrease the biosensor's performance over time. There are some essential factors for an ideal solid medium to use in Impedance spectroscopy. Before reliable and relevant measurements may be performed, the solid medium must be homogeneous and stable, with no changes in its properties over time. In addition, the qualities of the solid medium, such as its viscosity and conductivity, may influence the impedance measurements; therefore, it is essential to account for these factors when interpreting the results. In addition, the biosensor itself, such as the type of

immobilised biomolecules, and the design of the electrodes, can significantly impact the accuracy of the results.

Second, the results' stability might vary between each cleaning, especially after multiple uses of the sensor. It might be suggested to use a dryer to accelerate the dehydration of the sensor. It might be a thermal drier or chemical such as dry nitrogen gas.

Alternatively, options to eliminate buffers might be considered to increase the practicality of the wearable device, which can be helpful in either impedance spectroscopy or the electrochemical cyclic voltammetric method. For example, in CV methods, using a solid-state sensing material, such as a thin film of molecularly imprinted polymers (MIPs) placed on the electrode surface, is one technique to do CV with a solid medium. This avoids the requirement for a buffer by providing a specific binding site for cortisol. As a result, cortisol might directly interact with the sensing material, and the ensuing electrochemical signal can be used to detect the cortisol's presence.

Second, the consistency of the data may change between cleanings, particularly after many applications of the sensor. Using a drier or heater may be suggested to expedite the sensor's dehydration. This could be a thermal dryer or a chemical like dry nitrogen gas.

Third, the best cleaning duration has yet to be determined. Notably, the longer cleaning time causes the resistance to rise. However, it is also parallel to the surface evaporation of the liquid. In the interim, observing the biosensor response during the initial test is the only way to determine whether the cleaning operation was successful. The operation is successful if the response is modest because fewer electrons are stored in the dialectic zone.

7.2.1 Future Directions

The current study reveals that electrochemical impedance spectroscopy is a viable choice for wearable devices as opposed to the traditional electrochemical methods used for cortisol detection. However, the approach can also detect sweat cortisol as a home kit device. In addition, it can be a valuable instrument for merging wearable multi-biosensor systems to detect emotional stress. These biosensors can sense emotional occurrences. Adding a stress biomarker such as cortisol to the monitored biomarkers would boost the platform's accuracy.

Also, the measurement of cortisol in real sweat has yet to be conducted due to the pandemic restrictions. Therefore, it might be recommended to conduct further research to measure cortisol in human sweat. Furthermore, it is recommended to conduct the trials after developing the biosensor by lowering the resistivity factor to suit the human sweating rate. Otherwise, it would be ideal to use sweat collection methods.

There are several things to consider when developing a wearable molecularly imprinted polymer (MIP) biosensor for impedance spectroscopy monitoring. The shape and size of the MIP biosensor should be tailored for integration into a wearable system. In addition, the MIP biosensor should be compact, comfortable, and lightweight enough to be worn all day without causing discomfort and durable enough to withstand repeated use. The sensitivity and accuracy of the measurement will be determined by the specifics of the MIP biosensor's design and the assay settings. The MIP biosensor must be sensitive enough to detect the target molecule at the required concentrations. In addition, the measurement must be consistent and repeatable. The consistency of the experimental conditions impacts the accuracy and repeatability of measurement. Therefore, protecting the wearable MIP biosensor from environmental conditions that could alter the reading is crucial. These aspects include temperature, humidity, and mechanical stress. Wearable MIP biosensors should be user-friendly and comfortable to use for extended periods. The MIP biosensor could be made to feel more at home on the skin by using soft and flexible materials. The MIP biosensor that may be worn should be simple to use and maintain. Therefore, the MIP biosensor might be designed with simplicity and ease of use, including features that simplify

routine upkeep, like cleaning and changing the recognition element. The wearable MIP biosensor should be robust and withstand everyday use without damage.

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Appendix A:

Identification of Suitable Biomarkers for Stress and Emotion Detection for Future Personal Affective Wearable Sensors

biosensors

MDPI

Review

Identification of Suitable Biomarkers for Stress and Emotion Detection for Future Personal Affective Wearable Sensors

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Abstract: Skin conductivity (i.e., sweat) forms the basis of many physiology-based emotion and stress detection systems. However, such systems typically do not detect the biomarkers present in sweat, and thus do not take advantage of the biological information in the sweat. Likewise, such systems do not detect the volatile organic components (VOC's) created under stressful conditions. This work presents a review into the current status of human emotional stress biomarkers and proposes the major potential biomarkers for future wearable sensors in affective systems. Emotional stress has been classified as a major contributor in several social problems, related to crime, health, the economy, and indeed quality of life. While blood cortisol tests, electroencephalography and physiological parameter methods are the gold standards for measuring stress; however, they are typically invasive or inconvenient and not suitable for wearable real-time stress monitoring. Alternatively, cortisol in biofluids and VOCs emitted from the skin appear to be practical and useful markers for sensors to detect emotional stress events. This work has identified antistress hormones and cortisol metabolites as the primary stress biomarkers that can be used in future sensors for wearable affective systems.

Keywords: stress; emotion; cortisol; volatile organic components; biomarkers; wearable sensors

1. Introduction

For many years, scientists have known that emotions can be communicated among animals by changing their body odors [1]. In stressful events, such as being injured or in life-threating situations, chemical biosignals are released from the skin to warn other animals to escape or to gather. For example, Valenta and Rigby [2] showed that rats can differentiate between stressed and relaxed rats using airborne odor. Therefore, it has been postulated that such effects may be extended to humans. Many experiments have been conducted to determine the role of human odors in emotional communication. Consequently, it is now known that humans can smell several emotions, including happiness [3], fear [4], and anger [5]. Indeed, Benderly [6] stated that "olfaction is our most emotional sense".

In addition to body odor, physiological changes (such as heart rate, skin conductivity, and oxygen saturation) in the human body occur as an emotional response. Hui and Sherratt [7] used physiological sensor data to detect emotional events based on the concept of emotional context awareness. Happy and Routray [8] used image processing to detect emotional states in facial expression. Li et al. [9] merged facial image processing with electroencephalography (EEG) for improved emotional state

detection, indicating that affective systems benefit from being multimodal. Yang et al. [10] demonstrated emotion detection through speech for AI-based home assistants. While there is a large research area around affective systems and their impact on emotion and stress, this paper will review the literature, specifically looking for identified biomarkers in sweat that could be used to improve future affective sensors' sensitivity or classification to detect stress and emotion.

In the modern era, stress has been identified as a significant factor that affects health, the economy, and quality of life [11–14]. Researchers have recognized the relationship between the emotions of an individual and their health [13], which in turn has raised the subject of recognizing emotional status through affective computing [15]. These emotions were classified by some researchers into six basic emotions, namely fear, disgust, joy, anger, sadness, and surprise [13]. Recently, stress has been added to the recognized emotion set, which can be defined as the feeling caused by emotional tension, which might happen in certain circumstances when one has to react to demand or pressure that does not match with knowledge and experience, or is over their capability [11,16]. In the modern world, stress is a crucial problem. For example, researchers have reported that a growing number of community violence cases are related to anger resulting from stressful experiences [5,17,18]. Furthermore, police officers who do not cope with stress and its consequences have been shown to have increased rates of post-traumatic stress disorder and increased aggression [17]. Also, stress has been shown to harm human health and plays a key role in diseases related to a mental disorder, such as anxiety [19] and seizures [14,20]. Because of these risky influencers of stress, researchers have focused on overcoming the issues and detecting stress as early as possible to prevent further development. Although the classic invasive blood cortisol tests are the gold standard for measuring stress, there are two major methods that have been used to detect stress noninvasively, either measuring brain waves via implementing EEG electrodes or utilizing biomedical tools to detect physiological biosignals, such as heart rate (HR), blood pressure (BP), and body temperature, and by using sweat sensors to measure skin conductivity (SC) [21]. In terms of device wearability, although EEG provides accurate readings and valuable information about the brain's states, its main disadvantage is that EEG electrodes must be attached to the scalp. which is reported to be inconvenient for users [22]. While SC sensors are common in emotion detection systems, they are mainly used for measuring skin conductivity rather than the electrochemical content of the sweat. Sweat's electrochemical contents, such as the stress hormone cortisol, and skin gases are significantly under-researched.

This review considers the current state of the art in the understanding of biomarkers present in sweat under stress and emotional events. We present the most recent electrochemical sweat markers and skin VOC studies to hypothesize potential stress biomarkers for future affective technology sensors.

Section 2 presents our research methodology. Stress sweating physiology and stress electrochemical biomarkers are discussed in Section 3. A review of gas emissions from the skin, known as volatile organic components (VOCs), during events of emotional stress is presented in Section 4. Results are presented in Section 5 by evaluating each biomarker in terms of wearability, availability, and potential and future directions. Section 6 discusses the implications of the work, while Section 7 concludes the work and provides a guideline for future research.

2. Method

The design and methods used for this structured review comply with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines [23]. Regarding eligibility criteria, we accepted all types of design and research outputs, and no restrictions were applied to samples. The followed PRISMA guidelines results are presented in Figure 1.

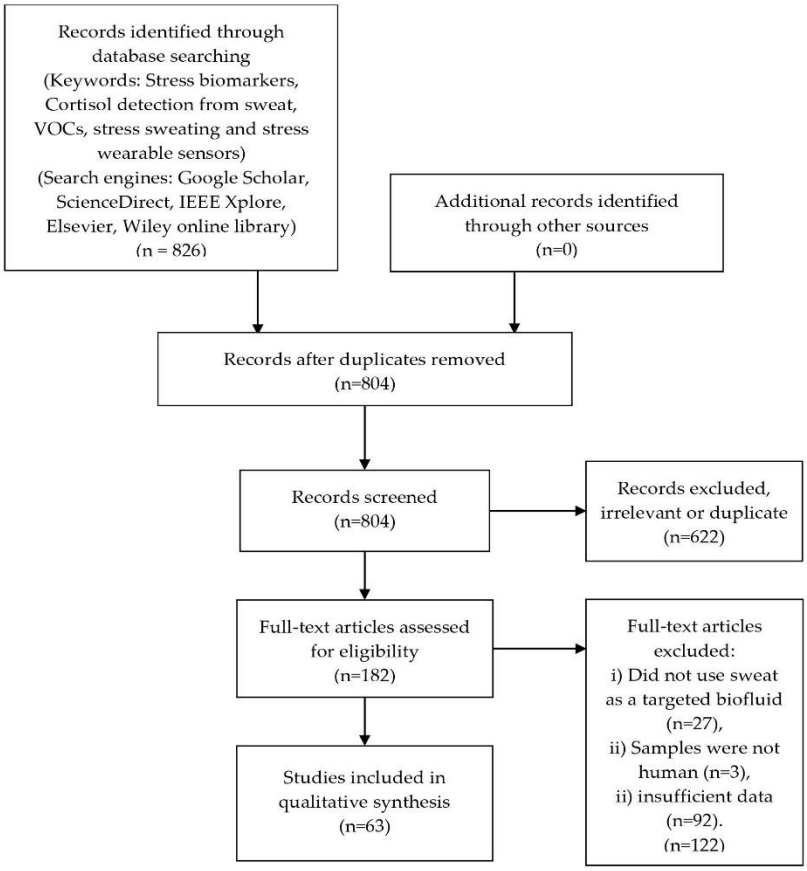

Figure 1. PRISMA process adopted and the results obtained in this review.

3. Sweat

3.1. Emotional Sweating Physiology

Perspiration's main role is to maintain the core temperature of the human body at optimum levels, which is important for survival, as increasing the core temperature to over 40 °C causes serious health issues and can lead to death. In other words, the main objective of sweating is the downregulation of the body's core temperature in high-temperature environments or under physiological stress. However, there are other major roles for sweating, including gustatory sweating and emotional sweating [24]. Regarding emotional sweating, this occurs as a physical reaction against emotive stimuli such as stress [25]. In an event of exposure to acute stress, the human body initiates several behavioral and physiological responses, known as the fight-or-flight response, which includes several connected activated mechanisms that enhance survival in events of danger and maintain homeostasis. The sympathetic nervous system reacts to acute stress by sending adrenaline and noradrenaline signals that

cause multiple physiological changes, such as increases in heart rate, blood pressure, and breathing rate [26].

In a slightly slower timeframe, the hypothalamic–pituitary–adrenocortical (HPA) axis is activated, resulting in a production of the stress hormone cortisol as a part of increasing the circulation of glucocorticoids [26]. Emotional sweat is produced on the entire surface of the skin, but it is concentrated on the palms, soles, and underarms. All of these responses are relative, and as such the level of response is based on several factors, including the nature of the stressor and the stressed person [25]. Sweat from the palms and soles is usually caused by emotive stimuli, not by environmental temperature [27]. In comparison to thermal sweating, which can be affected by ambient temperature, emotional sweat does not change in response to the surrounding environment temperature. It increases dependently and decreases during mental repose and sleep [24]. On the other hand, similar to thermal sweating, sole and palm emotional sweating involves the eccrine glands [27]. However, there is a lack of information regarding the central pathway of the eccrine glands, although some evidence has shown that the cortex and amygdala are involved [28]. Interestingly, emotional sweating of the axillary area does not occur before pubescence, suggesting apocrine and apoeccrine glands play key roles in axillary emotional sweating, as they are inactive before this stage [29]. Apocrine glands are activated by adrenergic stimulation and strongly respond to emotion [30]. However, the function of the secretion in these glands is unclear yet, although there is evidence that apocrine odors have similar effects to pheromones [4].

3.2. Electrochemical Biomarkers from the Sweat

The human sympathetic nervous system reacts to stress through many physical and emotional reactions, which are collectively termed the fight-or-flight response. This response is activated from the sympathetic nervous system and adrenal medulla by several mediators, such as noradrenaline, leading to the production of cortisol from the adrenal cortex [31] and adrenaline [32]. However, there are different types of stressful situations, including the fight-or-flight response, acute stress, and chronic stress, causing the human body to react in many ways. In this regard, other hormones are produced in events of stress, such as corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and urocortin [33]. In addition, in response to stress, the levels of many hormones, such as insulin and growth hormone, are altered to adapt to the new circumstances [34].

The salivary cortisol test has been identified as the most effective and promising noninvasive method to measure the cortisol level from biofluids [12] in concentrations ranging between 8.16 to 141.7 ng/m [35]. Most recently, sweat has started to be an attractive area of research for measuring cortisol [36]. In 2016, researchers developed a wearable device using nanosheets of zinc oxide (ZnO) to detect cortisol in sweat at concentrations of 1 to 200 ng/mL. The study used a thiol-based linker molecule to bind to the ZnO [37]. For low levels of cortisol volume detection, a portable cortisol sensor was developed using MoS2 sheets integrated into a nonporous flexible electrode system, as can be seen in Figure 2. The system succeeded in detecting volumes in the range of 1–5 µL. An affinity assay was designed, using MoS2 nanosheets operationalized with cortisol antibodies [38].

Most recently, CortiWatch, which is a wearable wristband with a watch shape, was developed for monitoring cortisol fluctuations within the physiological range (8–151 ng/mL) for 9 h. Although this device is a significant achievement in the field, it was designed to be disposed of after a low number of readings has been taken. The device has the potential to improve some medical applications, such as creating a circadian profile for a user and providing proof that self-monitoring of cortisol levels is possible [39]. Another recent study introduced an immunosensor that can detect cortisol and lactate using the label-free electrochemical chronoamperometric technique. This technique involves bioconjugation of cortisol and lactate antibodies with electro-reduced graphene oxide e-RGO, which is utilized as a synergetic platform for signal amplification. The prototype device can connect to smartphones via Bluetooth and can detect responses at concentrations as low as 0.1 ng/mL. In terms of selectivity, the device showed no cross-sensitivity between the two biomarkers or other components

present in sweat [40]. Additionally, another cortisol detection immunosensor was introduced in a study using a miniaturized potentiostat (M-P) chip (LMP91000) to perform a three-electrode range cyclic voltammetry (CV) measurement. The system succeeded in detecting cortisol in the physiological range, with a sensitivity level of 1.24 μM of cortisol [41]. Additionally, a four-channel electrochemical impedance spectroscopy (EIS) analyzer module was designed to detect cortisol in sweat. This module utilizes flexible chemi-impedance sensors and was constructed with three gold electrodes for wearability. It was developed to detect cortisol in an ultra-low volume of sweat (1–3 μL) using an antibody-based technique, as well as to measure other physiological parameters, namely pH and skin temperature [42].

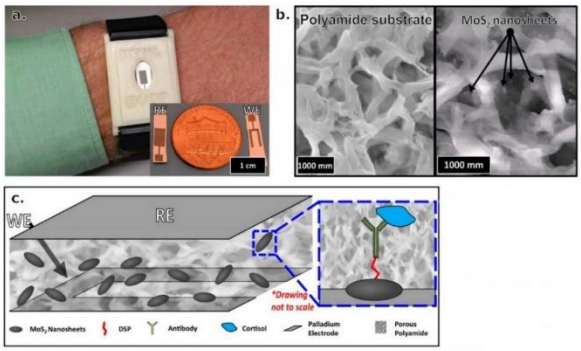

Figure 2. (**a**) Visualized wristband device prototype for monitoring cortisol in human sweat. (**b**) Scanning electron microscope (SEM) image of blank polyamide membrane on the left side, where MoS2 nanosheets were placed into porous polyamide membrane on the right side. (**c**) Stack of MoS2 nanosheets within a polyamide membrane sensing platform for cortisol detection. The blue box is a magnified picture of a nanosheet that presents the affinity assay for cortisol. Reproduced from Kinnamon et al. [38].

In addition to antibody-based methods, an alternative technique has been developed to detect cortisol in sweat. This technique is a colorimetric detection method based on the conjugation of cortisol selective aptamers with the surfaces of gold nanoparticles (AuNPs). The aptamers react with cortisol molecules present in the sweat, provoking their desorption from the surface of the gold nanoparticles, resulting in reddish color in AuNPs, as can be seen in Figure 3. The changes in color are due to the introduction of salt in the colloidal solution, which causes aggregation of AuNPs. The sensor detects within the physiological range of cortisol present in sweat, using the visual range of detection (1 ng/mL). There were no interactions with other biomarkers in sweat. Moreover, the aptamers technique has advantages over antibody-based methods in terms of stability, costs, and user-friendliness [43]. In addition, e-nose also has been involved in cortisol detection. A study used e-nose with in association with a pattern recognition software tool to detect a low concentration of cortisol (5 μM–50 μM) in sweat [44]. In addition, Parlak et al. [45] presented a new patch-type multifunctional layered biosensor that was developed to detect cortisol in sweat. A molecularly imprinted polymer (MIP)-based artificial recognition membrane was developed to interpose between semiconductor polymer channels, typically the poly(ethylenedioxythiophene)–poly (styrenesulfonate) (PEDOT–PSS) channel layer, and the sweat reservoir to control the molecular transport (which is selective to cortisol) immediately from the skin to the organic electrochemical transistor (OECT) sensing channel. This molecularly selective OECT (MS-OECT) showed physical and chemical stability at body temperature, as well as the ability to counteract physical deformation. The device succeeded in detecting cortisol at low concentrations (0.1–1 μM) and no errors were reported concerning selectivity and specificity. Finally, Mugo and Alberkant [46] introduced a flexible nonenzymatic biometric

molecularly imprinted electrochemical (MIP) sensor to detect cortisol in sweat. The sensor was fabricated using layer-by-layer (LbL) assembly and based on flexible poly(glycidylmethacryate-co ethylene glycol dimethacrylate)(poly (GMA-co-EGDMA)). The MIP was built to suit the human skin as a wearable device, as well as to be selective for cortisol detection in human sweat. The sample was collected from the forehead of one volunteer after exercise. The experiment was repeated for both a MIP sensor and a nonimprinted polymer (NIP), namely a cortisol-free labelled film that was polymerized similarly to the MIP but without the addition of cortisol. The selectivity of the sensor has been shown to be blind to other interfering sweat components. In terms of selectivity, the MIP sensor succeeded in detecting cortisol effectively in human sweat in the range of 10–66 ng/mL. However, the sensor has a limitation in terms of detecting cortisol at a lower range (2.0 ± 0.4 ng/mL). In comparison with the aptamers technique, the MIP technique is more economic and specific.

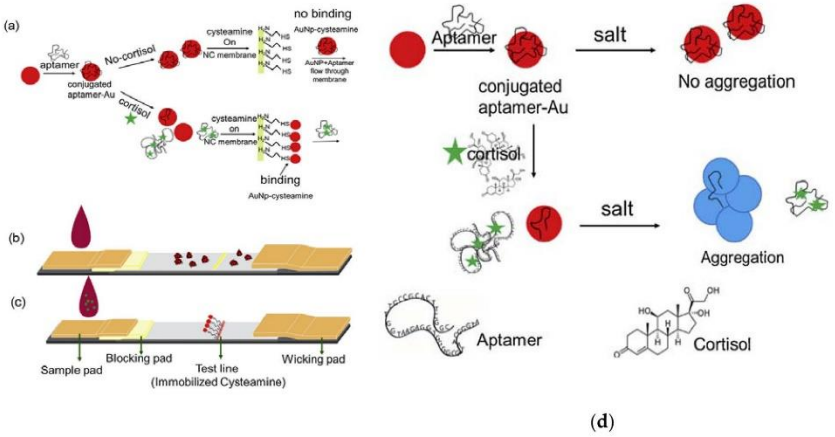

Figure 3. (**a**) Cortisol detection utilizing AuNP-aptamers: (**b**) negative control, for which there is no change in color in the absence of cortisol; (**c**) color change when cortisol is present; (**d**) process of cortisol effectiveness in releasing aptamers following salt aggregation. Reproduced from Dalirirad and Steckl [43].

Regarding alternative biomarkers, a study also discovered that cortisol's downstream metabolites (the 20α/β-DHCN) in the eccrine glands can be utilized as stress biomarkers in parallel with cortisol and cortisone [34]. These biomarkers have stability in terms of production in reaction to stress. The concentrations of cortisol and its metabolites were not altered by variables such as temperature and pH. The only concern is that concentrations were affected by the presence of other enzymes produced during a stress event [47]. However, the method was only used to detect these biomarkers in the laboratory, but it has the potential to target biomarkers in the future for wearable biosensor studies. Another aspect to be taken into consideration is the use of antistress hormones as biomarkers for stress as they are involved in the body's reaction to stress [33]. Oxytocin has been identified as an antistress mediator [48]. Recently, a biosensor was developed to detect oxytocin using Zn^{+} ions from biofluid [49]. However, the biosensor was developed to detect Zn ions and Cu ions in biofluids, and was not designed for stress detection. This raises the question of whether it could be modified to detect stress. Further feasibility studies are needed to address the advantages and disadvantages of this sensor and to compare it with the current options available for detecting stress.

4. Volatile Organic Components (VOCs)

The development of a noninvasive tool offering a significant level of selectivity and sensitivity with real-time operation is a challenging issue. For this reason, VOC sensing technology has been widely used in the medical field for several diseases that exhibit specific changes in the pattern of the VOCs of sweat [50]. Various gases are released from human bodies, including metabolic gases, while sweat VOCs and VOCs are produced by floral bacteria [51]. On the other hand, there is a lack of research on VOCs relating to human emotions, even though several studies have tested the role of sweat in human emotional interactions, such as fear sweat [52] and anger aggression [5]. These studies present the olfactory roles in emotional interactions, while the roles of chemical contents of emotional sweat had not been the focus of prior studies. Therefore, one study hypothesized that stress biomarkers are released from the skin in response to stress. The study used the trier social stress test (TSST) to measure stressors, a cortisol salivary concentration test as the gold standard for the study, and a survey as the result comparison tools. The gas analysis was performed by gas chromatography–mass spectrometry (GC/MS) system. The participants of this study were 30 females, as they are respond to TSST better than males. These subjects had a general anxiety tendency, which was evaluated using a physiological questionnaire. The subjects ranged between normal and high anxiety trait levels, reflecting the type of people who are likely to suffer from mental disorders as a result of stress. The study identified 6 stress biomarkers (1,2-ethanediol acetophenone, heptadecane, hexanedioic acid, dimethyl ester, benzyl alcohol, and benzothiazole) that were released from underarms of the samples [19]. Table 1 depicts the released amounts of the six stress VOCs that were identified as stress biomarkers in the skin. In the same vein, another study used a different methodology to identify stress VOCs. The paced auditory serial addition test (PASAT) was used as a stressor and sweat samples were collected from foreheads of 20 volunteers. The subjects were 10 males and 10 females between 19 and 26 years old. The samples were randomly separated into two sampling sessions. In the first session, subjects sat and listened to classical music. In the second session, subjects undertook the PASAT test. In addition, heart rate and blood pressure measurements were recorded. It was found that four stress biomarkers (benzoic acid, n-decanoic acid, a xylene isomer, and 3-carene) were present, as can be seen in Figure 4 [53]. Notably, the identified biomarkers from both studies were different. However, in terms of wearability, there are no commercial biosensors available to detect stress via VOCs, but a study did recommend a nanomaterial-based sensor array for future wearable biosensors for VOCs [54]. Unlike GC/MS, which identifies specific VOCs, this array relied on the collective pattern of VOCs.

Table 1. Released amount of volatile organic component (VOC) stress biomarkers. Reproduced from Tsukuda et al. [19]. AUC, area under the curve.

Compound	CAS No	*m/z*	Retention Time (min)	'Under Stress Task' vs. 'Relax1'		'Under Stress Task' vs. 'Relax2'	
				AUC Value	*p*-Value (Wilcoxon's Sign Rank Test)	AUC Value	*p*-Value (Wilcoxon's Sign Rank Test)
1,2-Ethanediol	107-21-1	33.1	25.6	0.82	<0.001	0.69	<0.001
Acetophenone	98-86-2	105	26.7	0.84	0.001 21	0.69	0.0019 23
Heptadecane	629-78-7	57.1	27.6	0.81	0.003 15	0.60	0.674 22
Hexanedionic acid, dimethyl ester	627-93-0	114.1	29.5	0.88	<0.001	0.74	0.0042
Benzyl alcohol	100-51-6	79.1	30.2	0.81	<0.001	0.75	<0.001
Benzothiazole	95-16-9	135	31.4	0.87	<0.001	0.66	0.153 65

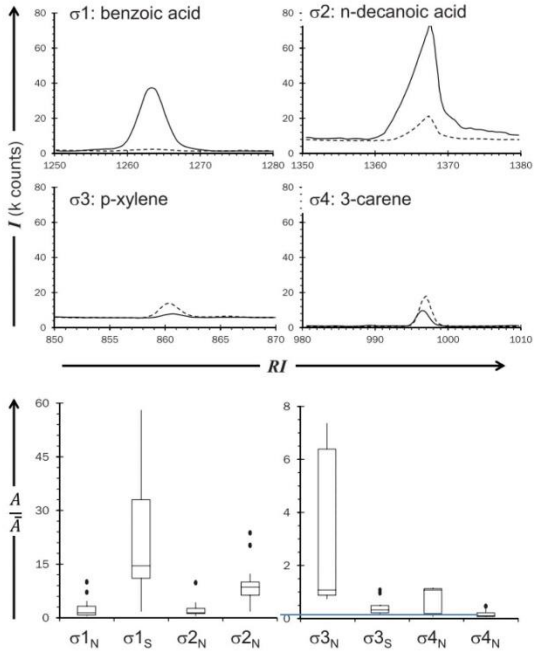

Figure 4. Overlaid extracted ion chromatograms from a sample from the four stress VOCs. Reproduced from Martin et al. [53].

5. Results

The results show a notable development in the field of electrochemical stress biosignals from sweat. Several methods have been utilized to detect cortisol, the main stress biomarker. In this regard, the antibody-based technique is the most common tool used to detect cortisol in sweat [37,39–41,55], while less commonly used techniques include the aptamer [43], e-nose [44,56], and MIP [45,46] techniques. In terms of sensitivity, all of the above-mentioned studies succeeded in detecting cortisol in its targeted range. However, different detection ranges were presented in the studies. The lowest levels of cortisol concentration detected were in the range 0.1 to 1.0 μM [45], while the only manufactured biosensor, CortiWatch, achieved a more modest level of detection, ranging from 1 to 150 ng/mL [39]. From the perspective of the placement of wearable sensor devices, it is an advantage that the eccrine glands are spread over the whole human body, as this offers a variety of placement options. In terms of selectivity, no reported errors were mentioned in cortisol biosensor studies.

Cortisol metabolites have the potential to be sensed as stress biomarkers in wearable devices. The current methods to detect them require sophisticated lab-based machines [47]. Further investigations are needed to create long-lasting sensors for wearable devices. Antistress hormones as stress biomarkers are also under-researched. However, a Zn^{+} ion biosensor has been developed to detect an antistress hormone called oxytocin in biological fluids for medical purposes [49]. From considering the literature, it is possible to recommend that more trial studies be conducted to detect the ranges and concentrations of biomarkers in sweat during a range of common stressful events, in order to further facilitate the capture of information needed in the design of biomarker sensors in future affective systems.

VOC technology is in the development stage. Two studies utilized different methodologies and found different stress biomarkers. The first study found changes in the concentrations of the biomarkers 2-hydroxy-1-phenylethanone, benzaldehyde, and 2-ethylhexan-1-ol in response to the stressor [53], while the second study found changes in the concentrations 1,2-ethanediol acetophenone, heptadecane, hexanedioic acid, dimethyl ester, benzyl alcohol, and benzothiazole [19]. However, in the first study, the biomarkers were found in the forehead samples, which might be produced from eccrine glands as cortisol metabolite VOCs, whereas in the second study the biomarkers were measured from apocrine glands (underarms). This difference might be the reason for the discrepancy between the experimental results obtained. Another possible reason is that the VOC biomarkers resulted from floral bacteria [19]. Further investigations are needed to understand the source of axillary VOCs, to also test the accuracy for both types of glands, and to test the performance of eccrine gland biomarkers at different places on the body.

Various signals have been identified as stress biomarkers. Table 2 summaries the results that were found in our research. Cortisol has been the most popular stress biomarker in sweat, with eight studies having targeted cortisol in the physiological range of sweat. Three techniques have been utilized to detect cortisol in experiments involving antibodies, aptamers, or e-nose technology. Antibody recognition methods including immunoassay and electrochemical immune sensing were utilized in five out of eight studies to detect cortisol [37,39–41,55]. These methods were effective in terms of specificity to cortisol molecules because of the nature of antibody–antigen immunochemistry [55]. CortiWatch [39], a cortisol wristband sensor, presents the antibody technique as an advanced step in this field. In terms of placement, the antibody-based methods detect cortisol from eccrine sweat, creating a promising future for cortisol detection technology, as eccrine glands are present on the whole surface of the body, which ensures the flexibility of manufacturing pervasive wearable devices. Alternatively, aptamer methods provide a visual, rapid detection method to detect cortisol in sweat [43]. The cortisol samples were, however, manufactured (i.e., no human body sample location was provided). Additionally, testing stress in real time has not been approved and finding suitable body placement locations for wearables have not been tested. Another cortisol detection method is e-nose, which "smells" the cortisol concentration in sweat vapors and uses additional pattern recognition tools to differentiate between stress events and quiet periods. Unlike the previous studies, sweat samples for this study were taken from the underarms of the samples, which means they were collected from apocrine glands. The sensitivity of the gas arrays increased directly with increasing cortisol concentration. However, a simplified wearable form of e-nose to detect cortisol concentration is not available. Samples were collected from apocrine glands (underarms), which could minimize the placements options, as apocrine glands are located in certain areas of the body, suggesting the potential for the development of wearable e-nose technology in "smart shirts" or armbands. Further studies are required to test e-nose technology for cortisol detection in eccrine glands, as succeeding in this would provide more fixable wearable options.

The combined response to stress of cortisol, its metabolites, and cortisone raises the idea of using multiparameters rather than only using cortisol, as all these markers are present in sweat within a measurable range. By using GC/MS techniques, all the markers can be separated from each other, and also from other components of sweat, then variable concentrations and patterns can be measured in stressful events [45,47]. The samples in these studies were collected from eccrine glands, which indicates flexibility in terms of wearable device developments in the future.

Table 2. Summary of stress biomarkers from the sweat or skin, methods used to measure them, places flexibility, wearable device availability, and potential devices for future works. MIP, molecularly imprinted polymer; GC/MS, gas chromatography–mass spectrometry.

Biomarkers	Methods	Place	Wearable Available	Potential Device
Cortisol [37,39–41,43–45,55,56]	Antibodies, aptamers, e-nose, and the molecularly selective organic electrochemical transistor	Eccrine glands (antibodies, aptamers and MIPs) Apocrine (e-nose)	Wrist band + patch	e-nose + Flexible
Cortisol metabolites [34,47]	In labs only	Eccrine glands	No	Flexible
Stress antihormones [49]	Zn+ ions	Eccrine glands	No	Flexible
VOCs (study 1) benzoic acid, n-decanoic acid, a xylene isomer, and 3-carene [53]	Lab (GC/MS)	Eccrine glands (or skin) (forehead)	No	E-nose/gas array sensors
VOCs (study 2) 1,2-Ethanediol Acetophenone Heptadecane Hexanedioic acid, dimethyl ester Benzyl alcohol Benzothiazole [19]	Lab (GC/MS)	Underarms skin or apocrine glands	No	e-nose/gas array sensors

Antistress hormones are present in human biofluids during stress [33] but utilizing them as stress biomarkers is significantly under-researched. However, the antistress hormone oxytocin has several functions and indeed a biosensor has been developed to detect it, but not in stress detection events [48]. That might suggest utilizing oxytocin as a stress biomarker in future studies. Additionally, because its presence in biofluid has been already confirmed, confirming its presence in emotional situations should be further tested.

In the first VOC study of its kind [53], benzoic acid, n-decanoic acid, a xylene isomer, and 3-carene were identified as stress biomarkers using the GC/MS lab technique. The sweat samples were collected from foreheads, which indicates that they are from the eccrine glands. In terms of wearability, this indicates the fixability of various places for monitoring. To detect VOCs in real time, e-nose and gas array sensors are commonly used, but unfortunately no device has been modified or developed to detect specific emotional VOCs. The second VOC study [19] found 1,2-ethanediol acetophenone heptadecane, hexanedioic acid, dimethyl ester, benzyl alcohol, and benzothiazole as stress biomarkers. While this work also used GC/MS tools, samples from the armpits (apocrine glands) were also collected. However, the source of VOCs collected from the axillary area is rather controversial, as they may be emitted by bacteria present in the collection area. Being limited to apocrine glands areas could minimize the options for developing wearable VOCs biosensors. Additionally, it is recommended to use human VOC sensors to detect the above-mentioned biomarkers using either e-nose or gas sensors.

6. Discussion

This paper has highlighted previous work, showing that the detection of sweat cortisol and VOCs emitted from the skin are effective methods for detecting stressful events, and have huge potential to supplement emotion detection systems in the future. Additionally, cortisol metabolites can be additional biomarkers to stress hormones that increase the efficiency of detecting emotional stress. Besides, antistress hormones can also potentially be used as stress biomarkers. Regarding cortisol detection using biochemical sensors, previous studies have shown three main methods, employing antibodies, aptamers, and MIPs. These methods have significant advantages over blood tests through classical laboratory techniques, as the latter requires a greater number of samples to be taken, consumes significantly more time, and needs trained staff to operate advanced tools [43]. In comparison between aptamers and antibody methods, aptamers are not rejected by the human immune system, as they are usually not considered foreign bodies, which makes them weakly immunogenic and toxic molecules, unlike antibodies that are highly immunogenic and toxic molecules [57]. Additionally,

aptamers have more thermal stability than antibodies because of the nature of oligonucleotide-based aptamers, which can maintain their structure, while protein-based antibodies lose their structure at high temperatures. Therefore, aptamers can be used in various assay conditions [58]. Additionally, the production of aptamers is a cost-efficient approach compared to antibody production and allows for easier modification for different chemical reactions [59,60]. Lastly, for future stress biomarkers that may have weak immune responses, such that antibodies cannot be generated or produced, aptamers can be recommended, as they can detect ligands that antibodies cannot recognize [61]. However, in a comparison between MIPS and aptamers, MIPs seem to be more economic [62] and more specific in terms of target binding [63]. Generally, MIPs have advantages over all other recognition systems, as they have high selectivity, are inexpensive, have accurate mechanisms, and are environmentally stable, as can be seen in Table 3. Therefore, due to these advantages, MIPs have been widely used in several industries, including in chemical sensors and drugs [45].

Table 3. Comparison between three cortisol detection techniques over several factors.

Factors/Techniques	Antibodies	Aptamers	MIP
Selectivity	High selectivity to cortisol—no errors have been reported	High selectivity to cortisol—no errors have been reported	High selectivity to cortisol—no errors have been reported
Sensitivity	In the physiological range	In the physiological range	The highest sensitivity (0.1 ng/mL)
Thermal stability	The lowest	High stability	The highest
Immune response	Can be rejected by the immune system	Cannot be rejected	Cannot be rejected
Cost	Expensive	Less expensive	Cheapest

However, the detection of cortisol directly from the sweat via e-nose technology is under-researched. In 2009, a study [44] showed a promising result, here e-nose detected stress situations by measuring the concentrations of cortisol and adrenaline in sweat; however, no further studies have been carried out on this. Alternatively, recent studies identified VOCs stress biomarkers emitted from the sweat or the skin during stress events [19,53]. However, the results of the two studies are controversial in many aspects. In the first study [53], samples were collected from eccrine glands (foreheads) and four stress biomarkers were found, while in the second study [19] no stress biomarkers were identified from the eccrine glands (palms), even though very similar methods (GC/MS) were used. This inconsistency raises the question of whether the eccrine glands are similar in different areas across the body. As some researchers have linked emotional sweating to the apocrine glands [19,64], it is also required to know if the eccrine glands produce emotional event VOCs. On the other hand, the source of stress VOCs identified by Tsukuda et al. [19] from the axillary area in the study is still unknown. The first possible source assumed was the apocrine gland, while the second possible source was floral bacteria. Addressing this issue may help find answers to the previous questions.

With respect to cortisol metabolites, they have been used as additional biomarkers for the stress hormones cortisol and cortisone for more accurate measurement. However, cortisol metabolites are only present 10 min after the production of cortisol in stressful events [47], which raises concerns regarding the effectiveness of utilizing them as biomarkers for acute stress, as this may require an immediate response. They may, however, be useful for less rapid stress situations or chronic stress conditions in mental health. Another challenge in this regard is that cortisol metabolites respond differently according to each individual, which suggests a need to develop techniques to deal with such individual differences [47].

With respect to antistress hormones, they are produced as a response to the production of stress hormones [33]. Oxytocin has been classified as an antistress hormone [48]. Although it has not yet been used as a stress indicator, its presence in biofluids has previously been detected [49]. More investigations are needed to check antistress hormone reliability as stress biomarkers, for example measuring the time between the production of stress hormones and antistress hormones. Additionally, although their presence in biofluid has been confirmed, their amounts in sweat must be confirmed in a measurable range.

Table 4 presents an analytical performance summary of the major biosensors reviewed in this work.

Table 4. Analytical performance summary of the major biosensors reviewed.

Reference	Stress Biomarker	Technique	Concentration	Volume	Within the Physiological Range of 8.16 to 141.7 ng/m? (Yes/No)
[37]	Cortisol	Cortisol antibodies	1 ng/mL to 200 ng/mL	N/A	Yes
[38]	Cortisol	Cortisol antibodies	N/A	1–5 µL	Yes
[39]	Cortisol	Cortisol antibodies	1 ng/mL to 150 ng/mL	N/A	Yes
[40]	Cortisol	Cortisol antibodies	0.1 ng/mL	N/A	Yes
[41]	Cortisol	Cortisol antibodies	1.24 µM	N/A	Yes
[42]	Cortisol	Cortisol antibodies	N/A	1–3 µL	Yes
[43]	Cortisol	Cortisol aptamers	1 ng/mL	N/A	Yes
[44]	Cortisol	E-nose	5 mL–50 mL	N/A	Yes
[45]	Cortisol	MIPs	0.1 µM–1 µM	N/A	Yes
[46]	Cortisol	MIPs	10 ng/mL–66 ng/mL	N/A	Yes
[19,53]	Stress VOCs	GC/MS	N/A	N/A	N/A

7. Conclusions

In this work, stress biomarkers were reviewed to present the current status of stress detection as an emotional event. In addition, potential biomarkers were also introduced for future studies. This paper has reviewed the electrochemical biomarkers of stress and highlights that cortisol is considered as a major stress biomarker because of its measurable presence in biofluids (sweat in this case), which makes it attractive to researchers. While most studies in this area have developed various methods of cortisol detection, this review also considered other possible stress biomarkers, including cortisol metabolites and antistress hormones, which are probably present in sweat as well. Another major focus of the work is volatile organic components (VOCs), which are have only just been considered in the most recent studies on stress detection. Studies has shown that there are a range of gasses emitted from different places on the skin, as demonstrated in various emotional stress tests. In several aspects, this field is still in the development stage. Firstly, the identified biomarkers from VOC studies are not yet coherent and different factors might be involved, such as stressors, placement, and types of glands. Secondly, all VOC experiments were measured in lab conditions; based on our knowledge, there are no currently wearable gas sensors available to sense human VOCs. However, some studies showed that e-nose or gas array sensors can smell environmental VOCs, as well as recognize human sweat cortisol concentrations by pattern recognition methods. It might be assumed that environmental VOCs biosensors can be modified to smell body odors. Also, pattern recognition for stress VOCs might be recommended for future studies.

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