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DETECTION OF VITAMIN D IN FOOD SUPPLEMENTS USING A SIMPLE MODIFICATION OF ELECTROCHEMICALLY REDUCED GRAPHENE OXIDE/SCREEN–PRINTED CARBON ELECTRODES

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ABSTRACT

Vitamin D (ergocalciferol and cholecalciferol) is a fat–soluble vitamin that is important for proper functioning of human metabolism which promotes calcium absorption for bone mineralization and prevent hypocalcemic tetany. Graphene is a two-dimensional nanomaterial offers superior properties of high surface area, high electrical conductance and great electrochemical properties. The development of electrochemically reduced graphene oxide (ERGO) for vitamin D detection is possible due to the non–polar property of ERGO and vitamin D. Initially, pre–treatment of the bare screen–printed carbon electrode (SPCE) was performed using cyclic voltammetry (CV) scan (–1.3 V to 0.4 V; 5 cycles) in 0.25 M potassium chloride (KCl) prior to the drop–casting of graphene oxide (GO) (5 µL, 0.7 mg/mL) on the sensor. Subsequently, a similar technique was applied on the GO/SPCE producing ERGO/SPCE. Several parameters of ERGO/SPCE have been optimized including GO concentration, CV cycle number and type of diluent. The developed sensor exhibits a linear detection of oxidation response of vitamin D, proportional to its concentration (0.50–8.0 mg/L; R^2=0.9849 for N=5). This result was qualitatively evaluated with one type of food supplement and the detection of vitamin D displays a promising result for hand–held future device application.

Keywords: Cholecalciferol, Electrochemically Reduced Graphene Oxide (ERGO), Ergocalciferol, Square–Wave Voltammetry (SWV), Vitamin D Supplement

INTRODUCTION

Vitamin D is a non–polar compound of fat–soluble vitamins group. Essentially, there are two types of vitamin D, namely ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). A certain sunlight wavelength (290–315 nm) exposure to vitamin D pre–cursor, ergosterol in plant such as mushroom, fungi and yeast capable to produce vitamin D_2 (1,2), whereas the similar radiation exposure to 7–dehydrocholesterol in human skin can naturally produce vitamin D_3 (3). This pathway represents around 90% of the natural sources of vitamin D. The remaining 10% of Vitamin D was reported to be developed in human body from food sources such as salmon, sardines, tuna and eggs. However, nowadays vitamin D can also be found in fortified food such as cow's milk, tofu, cereals and orange juice (4). Although vitamin D_2 contains an additional methyl group and a partially unsaturated phytyl chain (5) compared to vitamin D_3, it was evidently found that the biological activities for vitamin D_2 and vitamin D_3 is similar to each other (6).

The consumption of vitamin D helps the absorption of calcium and other minerals

in the human intestine (7,8). However, due to the low level of vitamin D in foodstuff (usually in the range of micrograms per 100g) (9–11), supplementation intake is a good option to reduce the risk of avitaminosis. Clinical studies have reported that the lack of vitamin D levels in patients' body can lead to severe diseases like cancer, cardiovascular disease, hypertension, depression and immunity (12,13) . Vitamin D deficiency was also linked to the bone diseases such as rickets in children and osteomalacia in adults as well as to non–bone related diseases (14). On the other hand, the excess of vitamin D intake through diet could cause vitamin D toxicity or hypercalcemia (15,16).

Despite the limited population data of vitamin D status, it was suggested that vitamin D deficiency may be widespread globally (17,18). It was generally presumed that sunlight exposure in low altitude countries was sufficient enough to prevent the vitamin D deficiency as well as in the industrialized countries, where vitamin D fortification has been implemented for years. However, the prevalence of vitamin D deficiency worldwide is still uncertain (17). Food fortification and supplementation may be considered as one approach to increase the vitamin D status in specific target group such as pregnant women and breastfeeding infants (18). In Malaysia, several studies have reported vitamin D deficiency among Malaysian especially to a few target groups such as pregnant woman, postmenopausal women and in adolescents (19–21). These research outcomes could lead to vitamin D supplementation recommendations. Besides that, the current trend of taking dietary supplements (that also contains vitamin D) was increasing in Malaysian adults (with high education level) due to the awareness of health benefits from the supplementation (22). Therefore, a continuous monitoring is needed so that the fortification of vitamin D in foods and supplements are within specified regulation range.

Currently, the only reliable method for vitamin D quantification in food matrices is liquid chromatography (23,24). This established method provides excellent sensitivity and highly specific. Nevertheless, they are also laborious, required a highly trained operator, time consuming of analysis, high organic solvent consumption and very costly. Hence, a simple method for the detection of vitamin D in food supplements will be able to tremendously reduce the analysis cost in the long run and provide an immediate result that is comparable to the current available detection methods. The utilization of electrochemical sensor has been a fast and easy–to–operate, and an alternative method to the conventional laboratory approaches. The use of small volumes of solvents and reagent, and cheaper consumable tremendously reduce the analysis cost as well as the instrumentation maintenance cost.

Graphene, a two–dimensional (2D) nanosheet of carbon atoms bound in hexagonal lattice structure (25), has gained its popularity in the sensor detection due to its large theoretical surface area (26), superior electrical conductance (27) and great electrochemical properties such as a wide potential window, low charge–transfer resistance, excellent electrochemical activity and fast electron transfer rate (28). Graphene oxide (GO), a derivation of graphene that contains functional groups such as epoxide, hydroxyl, carbonyl and carboxyl group on its basal plane (29), was employed in this study for the electrode modification purposes. Its oxide forms making the physical properties to be easily soluble in water, which simplify the modification procedures. However, this forms also distort the electrochemical activities of graphene in the GO compositions (30,31). Thus, the removal of oxygen is required if the graphene properties were to be retained. In small–scale, the simplest techniques for oxygen removal in the

GO were performed via electrochemical reduction.

There have been several previous reported studies for the detection of vitamin D via electrochemical techniques with different types of electrodes and modification approaches. These include the conventional three–electrode system of bare glassy carbon electrode (GCE) (5,32–34), gold–platinum bimetallic modified GCE (35), boron doped diamond electrode (36) and reduced–fullerene–C60 modified GCE with adsorbed copper–nickel bimetallic (37). However, the use of conventional electrode system was inconvenient due to the laborious procedure for analysis. Recently, the use of screen–printed carbon electrode (SPCE) modified with molecular imprinted polymer (MIP) for the detection of vitamin D has been reported (38). The modification tremendously increased the sensor selectivity toward vitamin D detection but the MIP preparation require thorough optimization and longer preparation time. Another study reported the use of graphene in nafion as ink for the fabrication of screen–printed electrode (39), which require instruments for designing and fabricating of electrodes. Thus, the aim of this study was to design a very straightforward and simple electrode preparation with the use of electrochemically reduced graphene oxide (ERGO) modification on the working electrode of SPCE (denoted as ERGO/SPCE) to detect the vitamin D through the non–polar interaction of ERGO and vitamin Ds.

MATERIALS AND METHODS

Reagents and Standards

Deionized water with 18.2 MΩ grade (Sartorius, Germany) was used throughout this study. Ergocalciferol (vitamin D_2), cholecalciferol (vitamin D_3), potassium perchlorate ($LiClO_4$) and N–Methyl–2–pyrrolidone (NMP) were purchased from Sigma–Aldrich (USA) whereas acetonitrile, ethanol, methanol, *n*–hexane and potassium chloride (KCl) were obtained from Merck (Germany). Potassium ferrocyanide ($K_4Fe(CN)_6$) was purchased from R & M Chemicals (Malaysia) while graphene oxide (4 mg/mL) in water dispersion solution was purchased from Graphenea (Spain). The SPCEs (model DRP–110D) used in this study for electrode modification were obtained from Dropsens (Spain).

Preparation of modified ERGO/SPCEs

The modified ERGO/SPCE was prepared and designed based on our previous study, with a slight modification of the above technique (40). First, the pre–treatment of the bare SPCE was done via cyclic voltammetry (CV) scans (–1.3 V to 0.4 V) for 5 cycles in 0.25 M KCl. Then, the modified electrodes were prepared by drop–casting the GO (0.7 mg/mL) on the working electrode of pre–treated SPCE with three electrode system (i.e., carbon as working and counter electrodes and silver as reference electrode). The GO modified SPCE (denoted as GO/SPCE) was dried in an oven for 30 minutes at 65 °C to strengthen the adhesion of GO film on the working electrode surface. The GO/SPCE was then electrochemically reduced using the same method during the pre–treatment of SPCE. After washing vigorously with adequate amount of deionized water, the newly formed ERGO/SPCE was dried in an oven for another 30 minutes at 65 °C. Finally, the modified electrodes were ready for analysis after cooling down to room temperature.

Optimization of modified ERGO/SPCEs

Several parameters were examined to maximize the oxidation signals of vitamin D produced by ERGO/SPCE including the GO concentration, CV cycle number, quiet time and type of solvent used for dissolving vitamin D_3. The optimization value was compared and selected based on the highest oxidation signal produced by vitamin D_3 at the concentration of 4.0 mg/L.

For the GO concentrations, 0.5, 0.7, 1.0, 1.3 and 1.5 mg/mL of GO were prepared individually in a 2–mL tube by adding 125, 175, 250, 325 and 375 µL of GO stock (4 mg/mL), respectively. The tubes were mixed after the addition of deionized water to the tubes for a final volume of 1000 µL.

The electrochemical reduction of GO/SPCE to ERGO/SPCE was optimized using several cycle numbers of CV (3, 5, 10, 15 and 20 cycles) at the potential range of –1.3 V to 0.4 V.

Prior to vitamin D_3 analysis using square–wave voltammetry (SWV) scan, 50 µL of standard solution was dropped on the modified ERGO/SPCE and let to settle at a certain time frame. In this study, the waiting time was referred as quiet time. The optimization of the quiet time was employed at 150, 300, 450, 600 and 750 s.

Several solvents were used for the optimization of type of solvent used to dissolve the vitamin D_3. The solvent and deionized water composition were fixed at 50% (v/v). The solvent used for the optimization include ethanol, acetonitrile, NMP and acetone.

Preparation of vitamin D standard

Vitamin D_3 was used throughout this study for the vitamin D detection and calibration. The stock solution of vitamin D_3 (1000 mg/L) was prepared by dissolving 10 mg of vitamin D_3 in 10 mL ethanol. The solution was mixed using vortex mixer for a few minutes until the vitamin D solute was completely dissolved. The stock of vitamin D_3 standard was kept in the freezer (–18 °C) when not in use. Similarly, the procedure was repeated for the preparation of vitamin D_2 stock (1000 mg/L).

Serial dilutions of vitamin D_3 stock was prepared for the five–calibration level of vitamin D_3 at 0.50, 1.0, 2.0, 4.0 and 8.0 mg/L. To prepare 0.50 mg/L of vitamin D_3, 0.5 µL of vitamin D_3 stock was transferred and mixed with 100 µL of $LiClO_3$ (1 M dissolved in ethanol) in a 2–mL empty tube. Then, 50% ethanol in deionized water was added to the solution to meet a final volume of 1000 µL. In separate 2–mL tube, the steps were repeated using 1.0, 2.0, 4.0 and 8.0 µL of vitamin D_3 stock for the preparation of respective to 1.0, 2.0, 4.0 and 8.0 mg/L vitamin D_3 working standard.

Sample Preparation

The supplement samples containing vitamin D labelling were obtained from Klang Valley, Malaysia. In each supplement samples, two (2) tablets were taken and grinded to a fine size using pestle and mortar. The grinded samples were then placed in the 15–mL centrifuge tubes containing 10 mL methanol and mixed for few minutes until the sample was well–mixed. Similarly, for spiked and recovery study, the procedure was repeated by adding known concentration of vitamin D_3 standard in the samples in a

different 15–mL centrifuge tubes. Next, the sample mixture was sonicated for 15 minutes to extract out the analytes from any other possible bonded materials in the supplement samples. Then, 1 mL of the supernatant was transferred to a new sample tube after the sample mixture was centrifuged (3,000 RPM) for 15 minutes.

Liquid–liquid extraction technique was employed by mixing 3 mL *n*–hexane to the 1 mL supernatant. The mixture was mixed for a few minutes using vortex mixer. Two (2) layers of solvent should form after the mixture was settled down for few seconds. The upper layer was then carefully pipetted (900 µL) to a new 2–mL tube. The upper layer solvent was dried using centrifugal vacuum concentrator (2,000 RPM) for 90 minutes.

Finally, the samples were reconstituted with 50% ethanol in deionized water containing 0.1M $LiClO_3$ prior to the electrochemical detection.

Electrochemical Detection

The prepared samples were dropped (50 µL of sample volume) onto the modified ERGO/SPCE surface. Square–wave voltammetry (SWV) scan was applied from 0.2 V to 1.0 V using Metrohm Autolab M101 (Sweden) after letting the samples to settle (referred as quiet time) for 10 minutes. Throughout the study, the SWV scanning parameters were specifically set according to the following value: scan range from 0.20 V to 1.0 V; step potential at 0.01 V; amplitude at 0.1 V and frequency at 10 Hz.

RESULTS AND DISCUSSION

Electrochemical characterization of vitamin D

The behavior of vitamin D was investigated using CV scan with applied voltage from –1.3 V to 0.4 V in the presence of supporting electrolyte of 0.1 M $LiClO_3$ in 50% (v/v) ethanol and water composition (Figure 1) on ERGO/SPCE electrode. Figure 1A shows that the blank supporting electrolyte was free from any electrochemical activity. After the addition of vitamin D_2 (4.1 mg/L; Figure 1B) and D_3 (4.0 mg/L; Figure 1C), an oxidation potential at 0.73 V with no reduction potential appeared in the applied potential range for both vitamins. This suggested that vitamin D_2 and D_3 only possess an irreversible reaction following their oxidation, forming a non–active product. The irreversible mechanism only occurs when vitamin D was mixed in the water–organic mixture, whereas reversible reaction was observed when vitamin D was dissolved in pure organic solvent containing supporting electrolyte (33).

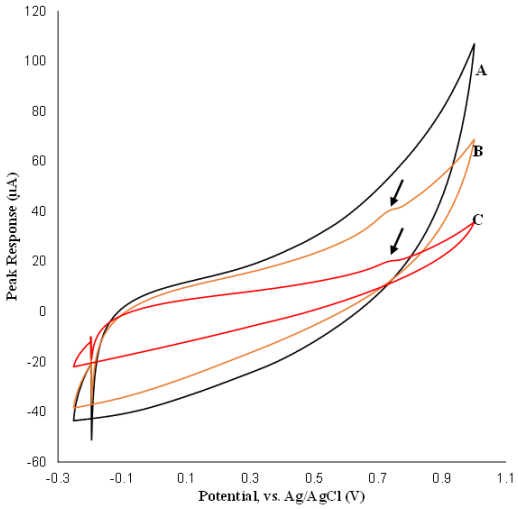

Figure 1. Overlay of CV scans (–1.3 V to 0.4 V) on ERGO/SPCE electrodes for blank supporting electrolyte (0.1 M $LiClO_4$ in 50% ethanol and deionized water mixture) (A), vitamin D_2 in supporting electrolyte (B) and vitamin D_3 in supporting electrolyte (C). It was observed that the oxidation peak appeared in (B) and (C) at 0.73 V of peak potential vs. Ag/AgCl.

Further study was done for both vitamin D_2 and D_3 using SWV (0.20 to 1.0 V) to observe the oxidation peak response (Figure 2). At equivalent molarity, both vitamin D_2 and D_3 appeared at the same peak potential and had similar peak intensity. The intensity comparison of vitamin D_2 and D_3 were also repeated several times (n=3) and it was found that the intensity ratio of vitamin D_2 (4.1 mg/L) to vitamin D_3 (4.0 mg/L) was 0.86 (Figure 2 inset). This indicates that both vitamins do have similar electrochemical properties which can be further supported by the similarity of their chemical structure on the three conjugated double bonds from C8 to C19 that contains high electron density. That was the main reason for their electrochemical activity causing the compounds oxidation reactions (33). Thus, vitamin D_3 was chosen and to be regarded as total vitamin D detection in the further investigation.

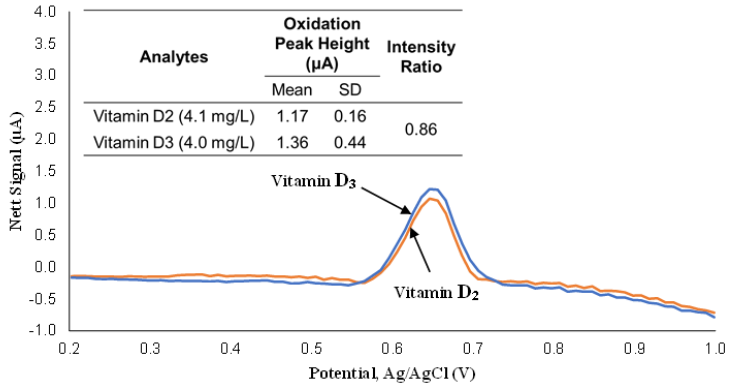

Figure 2: SWV scan (0.2 V to 1.0 V) overlay for vitamin D_2 (4.1 mg/L) and D_3 (4.0 mg/L), dissolved in 50% ethanol and deionized water mixture that contains 0.1 M $LiClO_4$. It was noted that both oxidation peak produced similar intensity.

Optimization of modified ERGO/SPCEs

The SPCE is prone to have impurities on the carbon working electrode due to its long–time exposure to the air which introduces unwanted oxygen–containing functional groups that would lead to the adsorption of polar molecule. This phenomenon could partially blocked the electrode active site thus reducing the electrode sensitivity (41). Therefore, an additional step of pre–treatment was needed to improve the sensitivity of SPCE. In our study, the pre–treatment using CV scan (–1.3 V to 0.4 V; 5cycles) was preferred due to the method's simplicity and *in–situ* approach. Based on the overlay results, the SWV scan (0.2 V to 1.0 V) of vitamin D_3 (4.0 mg/L) on the modified ERGO/SPCE was increased as much as 2.1–fold when the pre–treatment procedure was applied compared to the modified electrodes without the pre–treatment (Figure 3).

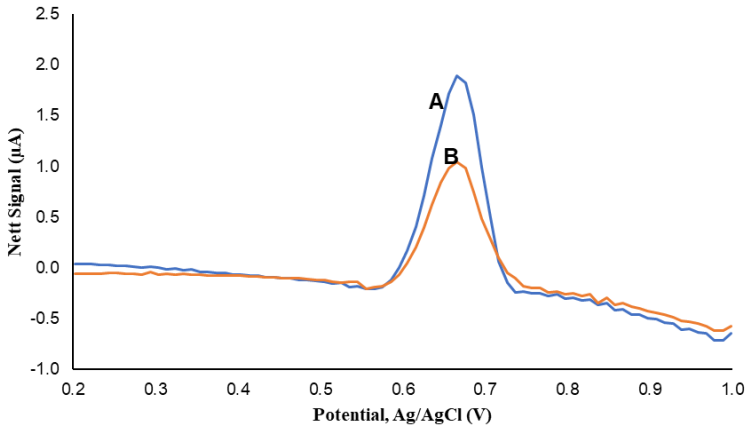

Figure 3. The effect of bare SPCE pre–treatment was illustrated in the overlay of SWV scan (0.2 V to 1.0 V) of vitamin D_3 at 4.0 mg/L, where pre–treated bare electrode of ERGO/SPCE produced 2.1–fold higher signal in (A) compared to the un–pretreated bare electrode of ERGO/SPCE in (B).

The modification of ERGO/SPCE was further optimized using several parameters including the GO concentration to form GO film on GO/SPCE, the CV cycle numbers during the electrochemical reduction of ERGO/SPCE, the quiet time applied after the sample was dropped on the modified electrode and the type of solvent to dissolved vitamin D_3 at 50% (v/v) solvent to deionized water composition. These parameters were investigated by comparing the vitamin D_3 oxidation signal, while biasing the voltage of SWV scan at 0.2 V to 1.0 V. Otherwise stated, the parameters used during optimization were as follows: GO concentration at 0.7 mg/mL; CV cycle number at 5 cycles; quiet time at 600 s; and the type of solvent used was ethanol–deionized water at 50% (v/v).

For the effect of GO concentration, the highest oxidation signal was observed at concentration 0.7 mg/mL compared to the other concentrations which were 0.5, 1.0 1.3 and 1.5 mg/mL (Figure 4A). It was shown that the vitamin D_3 signal was absent at the GO concentration of 0.5 mg/mL and the signal only appeared at the GO concentration of 0.7 mg/mL and onwards. However, lower signals and plateau pattern were observed after 0.7 mg/mL because of the over–concentration of GO, leaving more residual oxygen–containing functional groups which was ineffective for electron transfer (42) and would require more cycles of CV to further electrochemically reduced the GO. Thus, 0.7 mg/mL

concentration was selected as the optimum GO concentration.

A various number of CV cycles (3, 5, 10, 15 and 20) were done for the electrochemical reduction process of ERGO/SPCE and it was shown that the highest signal appeared when the CV cycle number was set at 5 cycles (Figure 4B). This indicates that 5 cycles were the best number of cycles to removes functional groups such as epoxy and hydroxyl that was located on the basal plane of the film, which contributed for the low conductivity of GO (42). Furthermore, increased in number of cycles only further reduced the other functional groups on the film edge such as carboxyl, carbonyl and ester which have less influence on the ERGO conductivity (43), as well as introduced the formation of new reduced functional group that might further insulate the ERGO film causing the observed of vitamin D_3 signal to drops further (44). Therefore, the CV cycle number was fixed at 5 cycles.

The study of quiet time was explored to increase the sensitivity of vitamin D_3 using the developed sensor. Several time frames were selected for the study including 150, 300, 450, 600 and 750 s. Based on the observed data (Figure 4C), longer quiet time produces higher signal intensity which indicates that longer time was required for the vitamin D to completely reach the working electrode surface. This was due to the dependency of vitamin D_3 interaction toward ERGO/SPCE was through non–polar and non–polar interaction. It was reported that vitamin D_3 possess a diffusion–controlled reaction [32]. The highest vitamin D_3 signal was observed when 600 s and 750 s of quiet time were employed. In this instance, the 600 s of quiet time was chosen for further characterization.

Generally, vitamin D can be easily dissolved in non–polar organic solvent due to the vitamin D non–polar property. However, since the method requires a quiet time step before the scanning was done, the solvent composition for dissolving the samples was fixed at 50% (v/v) solvent and deionized water. This procedure is conducted to prevent the solvent from easily evaporated to the environment that might cause harm to the operator. Thus, several types of solvents were investigated at the composition of 50% (v/v) with deionized water (Figure 4D). This investigation established that the best solvent for this method was ethanol–water mixture. The lowest signal response of vitamin D was produced when dissolved in NMP–water mixture. In fact, NMP is known to be a favorable solvent at dissolving wide range of product and it is non–volatile. However, its polar property makes it less compatible with a non–polar compound, such as vitamin D.

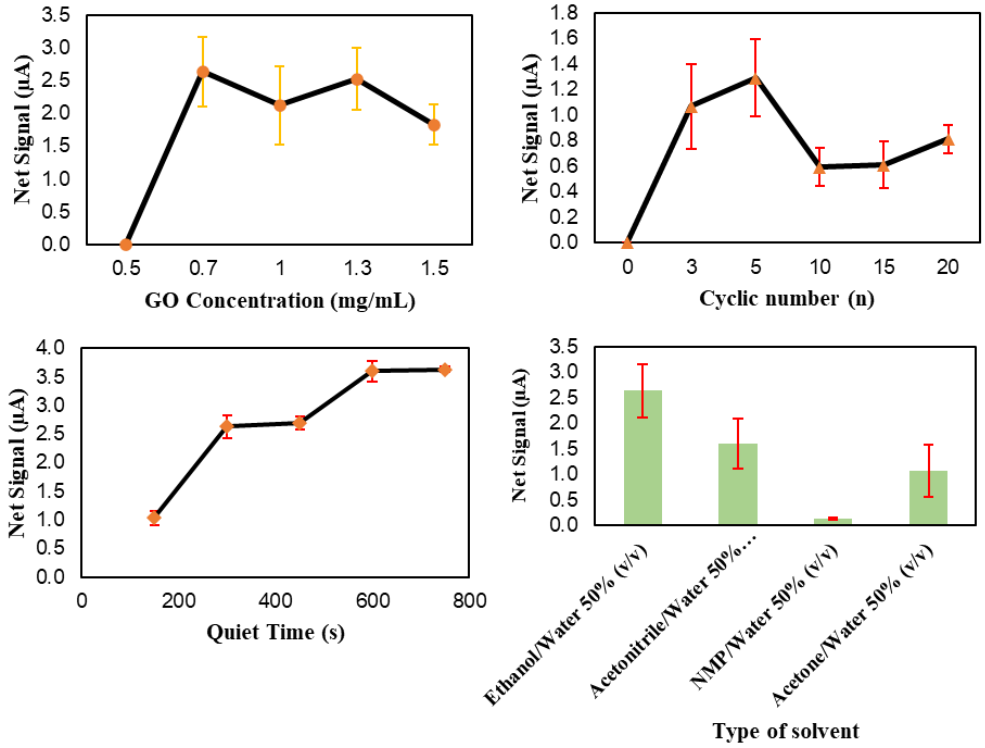

Figure 4. Compilation of optimized data for the response signal of vitamin D3 (in 0.1 M $LiClO_4$) towards the effect of GO concentration (A), the cycle number of CV during the electrochemically reduction of ERGO/SPCE (B), the quiet time before the SWV scan were done (C) and the type of solvent used to dissolve vitamin D_3 (D). The SWV scan range from 0.20 V to 1.0 V; step potential at 0.01 V; amplitude at 0.1 V and frequency at 10 Hz.

Vitamin D calibration plot

The construction of the calibration based on the oxidation peak response of vitamin D_3 was shown in Figure 5 and it revealed that, under optimum condition, the vitamin D_3 response was linearly proportional to its concentration ranging from 0.50 to 8.0 mg/L (R^2 = 0.9849; N=5) with LOD of 0.97 mg/L and LOQ of 2.95 mg/L. The fact that the calculated LOD was higher than the lowest level of calibration point was due to the high standard deviation when the scans were repeated. The possible reason for this was the adhesion of the ERGO film was not strong enough to be physically adsorbed on the working electrode surface of bare SPCE. Subsequently, certain parts of the film tend to be easily detached from the surface when the 50% (v/v) ethanol–deionized water mixture composition from the samples placed onto the modified ERGO/SPCE for over 10 minutes exposure, thus affecting the electrode sensitivity. According to reported studies, a simple linear regression can be correlated through coefficient of determination (R^2) where value of nearest to 1 give the best linear correlation (45,46). Thus, a good and acceptable calibration range was still observed (R^2=0.9849).

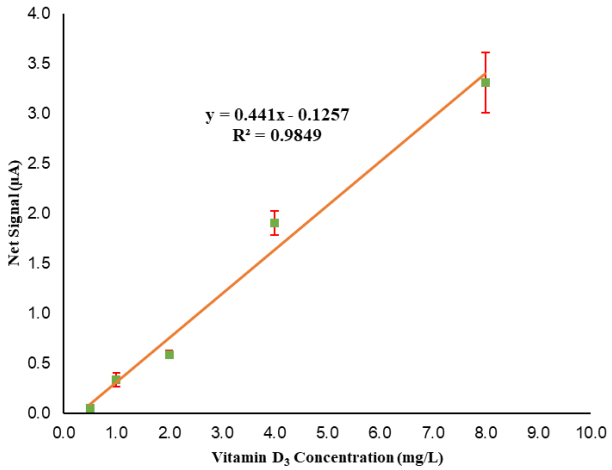

Figure 5. The plot of vitamin D_3 calibration from 0.50 to 8.0 mg/L in 50% (v/v) ethanol–deionized water composition containing 0.1 M $LiClO_4$.

Detection of vitamin D in supplement samples

One food supplement sample was analyzed qualitatively (n=3) using ERGO/SPCE sensor and it was found that the oxidation peak response of the sample appeared around the same peak potential of vitamin D_3 (Figure 6), indicating that the vitamin D was successfully detected by ERGO/SPCE sensor in real samples. However, further preliminary testing using spiked sample with 1.0 mg/L vitamin D_3 had shown a poor sample recovery ranging from 37% to 95% (data not shown). An extensive optimization of sample extraction had to be investigated in order to remove the co–extracted compounds that caused the signal interferences. Moreover, sample digestion steps for fat removal might increase the sample recovery. Also, more samples had to be analyzed and quantified to determine and validate the detection capability of the modified sensor.

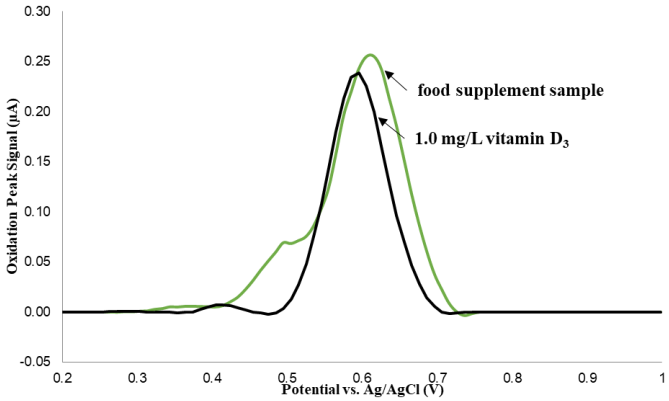

Figure 6. The overlay SWV scan of food supplement sample and 1.0 mg/L vitamin D_3, both in 50% (v/v) ethanol–deionized water mixture containing 0.1 M $LiClO_4$.

CONCLUSION

Since the detection of vitamin D_2 and D_3 were at the same peak potential and intensity, the concentration of vitamin D_3 can be considered as a total concentration of vitamin D. At optimized condition, vitamin D_3 is successfully detected in food supplement sample using a simple modification of ERGO/SPCE. The current sensor modification enables a simpler and straightforward step of analysis that require only 50 µL drop of samples needed. It also does not require electrode fabrication due to the *in–situ* electrochemical modification of commercial SPCE. However, an intensive investigation is needed in the future in order to build a stronger adhesion of ERGO film on the SPCE. This is because a stronger adhesion could improve and enhance the devices' sensitivity, thus reducing the quiet time before the analysis. Similarly, a digestion technique for fat removal might be helpful to increase the sample recovery in the spike–recovery study.

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