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Electrochemical determination of estradiol using a thin film containing reduced graphene oxide and dihexadecylphosphate

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Abstract
Graphene is a material that has attracted attention with regard to sensing and biosensing applications in recent years. Here, we report a novel treatment (using ultrasonic bath and ultrasonic tip) to obtain graphene oxide (GO) and a new stable conducting film using reduced graphene oxide (RGO) and dihexadecylphosphate film (DHP). The RGO was obtained by chemical exfoliation and it was reduced using NaBH4. Subsequently, RGO–DHP dispersion was prepared and it was dropped onto a glassy carbon electrode by casting technique. The electrode was characterized by cyclic voltammetry and electrochemical spectroscopy impedance. The voltammetric behavior of the RGO–DHP/GC electrode in the presence of estradiol was studied, and the results reported an irreversible oxidation peak current at 0.6 V. Under the optimal experimental conditions, using linear sweep adsorptive stripping voltammetry, the detection limit obtained for this hormone was $7.7 \times 10^{-5}$ mol L$^{-1}$. The proposed electrode can be attractive for applications as electrochemical sensors and biosensors.

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Introduction

Carbon nanostructured materials have been extensively used in sensing and biosensing applications in recent years [1–5]. For example, electrochemical sensors for pharmaceuticals/biological analysis [6–15] using pristine or modified nanomaterials have been proposed, which the modification of electrode can provide(s), e.g., increase in the analytical signal or improves the selectivity. Ensafi et al. have proposed a SiO$_2$–Al$_2$O$_3$ mixed-oxide electrode modified with Mn nanoparticles for oxidation of captoril. They have obtained a detection limit of 0.095 mmol L$^{-1}$ and have determined this compound in samples such as pharmaceutical and human urine [11]. Karimi-Maleh et al. have constructed a multiwalled carbon paste electrode based on NiO–carbon nanotubes nanocomposite and an anthracene-diol modifier for simultaneous determination of cystone, nicotinamide adenine dinucleotide, and folic acid in biological and pharmaceutical samples, which have presented a detection limits of 0.007, 0.6, and 0.9 mmol L$^{-1}$, respectively [6]. Moradi et al. proposed a sensor using FePt particles, multi-walled carbon nanotubes and an amide ligand as a mediator for simultaneous determination of three organic compounds in biological samples [10]. Detection limits of 0.05, 0.8 and 1.0 µmol L$^{-1}$ were achieved for glutathione, nicotinamide adenine dinucleotide and tryptophan, respectively.

Graphene-based materials have been used in several different types of applications, such as hydrogen storage [16], solar cells [17], sensors [18], and biosensors [19]. The applications of this material are related to their interesting properties, which include a high surface area [20], excellent electric conductivity [20], and strong mechanical strength [21]. The synthesis of graphene sheets remains a challenge, and several different methods have been proposed to prepare this material [22–26]. Novoselov et al. were the first to prepare graphene sheets by the exfoliation of pyrolytic graphite, which is also known as the scotch-tape method [27]. Other methods to obtain graphene include thermal decomposition under ultra-high vacuum (UHV) conditions [28], chemical vapor deposition (CVD) growth on metal substrates, substrate-free CVD [29], epitaxial growth in SiC [25] and the chemical exfoliation [30].

The chemical exfoliation of graphite utilizes oxidizing reagents (e.g., sulfuric acid, potassium permanganate and hydrogen peroxide) to obtain graphene oxide (GO). GO is a form of graphene that has emerged as an important derivative of graphene and can be reduced in presence of a reducing agent [30], including hydrazine, ascorbic acid or sodium borohydride. In this regard, reduced graphene oxide (RGO) has been used in electroanalysis [31]. The dispersion of graphene, GO or RGO in water is an important issue for the fabrication of many graphene-based devices [32], including electrochemical biosensors. To maintain the properties of individual graphene sheets, it is necessary to maintain stable suspensions of RGO in aqueous solutions. In this context, there is a need to develop procedures for directly dispersing relatively pure graphene sheets in aqueous solutions [33–35]. Some compounds that have been used to prepare graphene dispersions include poly(diallyldimethylammonium chloride) (PDDA), poly(ethylenimine) (PEI), poly(sodium styrenesulfonate) (PSS), poly(allylamine hydrochloride) (PAH), poly(acrylic acid) (PAA), and sodium dodecyl sulfate (SDS). Tummala et al. studied the effects of the structural properties of SDS on graphene [36]. Tang et al. proposed the preparation of graphene nanosheets from natural graphite modified with the cationic surfactant...
cetyltrimethylammonium bromide (CTAB) and the anionic SDS [37]. Li et al. prepared a hybrid material using graphene modified with gold nanoparticles in the presence of SDS [38]. In addition to SDS, dihexadecylphosphate (DHP) is a type of surfactant that has been used as a dispersant in film development for electroanalysis [39,40]. As observed by Janegitz and co-workers, DHP can produce a stable and uniform film using carbon nanotubes [4]. Thus, DHP is an alternative material for producing films using carbon nanomaterials as electrodes.

Estradiol (17β-estradiol) is a natural steroid estrogen that is important in many physiological processes and influences the development of sexual characteristics [4] (Fig. 1). Estradiol deficiency can cause diseases such as hyperandrogenism, cancer, heart disease, osteoporosis, and menopausal symptoms. The detection of estradiol is important in the prevention and treatment of related diseases that occur because of hormone dysfunction. The electrochemical determination of estradiol has been reported by different methods [41–46].

Here, we describe the preparation of GO by chemical exfoliation using a new treatment (ultrasonic bath and ultrasonic tip) followed by the reduction of GO using SDS and NaBH4. Then, RGO was immobilized on glassy carbon electrode (GCE) with DHP and used for estradiol determination in urine samples.

2. Experimental

2.1. Chemicals and reagents

Graphite was obtained from Nacional Grafito (Brazil) (<180 μm 99.5%). Ethanol (>99.5%), SDS, DHP and 17β-estradiol were obtained from Sigma. All other chemicals were of analytical grade. A 1.0 × 10⁻² mol L⁻¹ estradiol stock solution was prepared in ethanol. Phosphate buffer saline (PBS) solution (pH 7.0) was prepared using NaCl, KCl, Na₂HPO₄ and NaH₂PO₄. All solutions were prepared with Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm). PBS was employed as the supporting electrolyte in all electrochemical measurements.

2.2. Apparatus

Ultraviolet–visible spectroscopy (UV–Vis) and Fourier-Transform Infrared (FTIR) spectroscopies were carried out using a Hitachi U2001 and a Bomem (Hartmann & Braun) MB-102 spectrophotometer, respectively. The charges of the RGO solutions were measured using a Zetasizer Nano ZS (Malvern) and a MPT-2 Titrator. The scanning electron microscopy (SEM) images were obtained by JEOL JSM-6510 equipment with electron beam energy of 15 keV. The voltammetric measurements were performed using a three-electrode system: GCE modified with RGO–DHP/GCE as a working electrode, a platinum plate as a counter electrode and Ag/AgCl (3.0 mol L⁻¹ KCl) reference electrode. The electrochemical measurements were conducted using an Autolab Eco Chemie PGSTAT12 potentiostat/galvanostat. Cyclic and linear sweep voltammetric measurements were recorded using a 10.0 mL electrochemical cell at room temperature.

2.3. Preparation of GO

GO was prepared from graphite powder by modified Hummer method, as described previously [30,47,48]. In addition to this oxidation, we added a treatment to increase the effectiveness of exfoliation and to remove impurities. The GO dispersion was taken in an ultrasonic bath (80 W) for 30 min, and, it was subsequently sonicated with an ultrasonic tip (450 W, 25% amplitude and a 15 s pulse) for 1 h. Following, the GO dispersion was centrifuged at 4000 rpm for 2 h to remove the supernatant. After, this dispersion was added to a dialysis membrane, maintained for one week to remove impurities and, then, stored at 4 °C.

2.4. Reduction of the GO and preparation of working electrode

Ten milliliters of GO (1.0 mg mL⁻¹) dispersion and 10 mg of SDS were ultrasonicated (80 W) in a flask to produce a homogenous solution. Concentrated NH₃ was added until the pH reached 10; the solution was deoxygenated using N₂ for 15 min and, then, sodium borohydride (0.15 mol L⁻¹) was added over 5 min under vigorous stirring. The time required for reduction and the appropriate concentration of NaBH₄ were determined using 1.0 mg GO and 1.0 mg SDS in 1.0 mL of water and were verified by UV spectroscopy. The best results were obtained after 60 min, so, this time was used for further studies. The concentration of NaBH₄ was examined over a range from 0.02 to 0.3 mol L⁻¹. The highest absorbance values were obtained using 0.15 mol L⁻¹ NaBH₄. Therefore, 0.15 mol L⁻¹ NaBH₄ was chosen for further studies. The reaction mixture was heated to 80 °C for 1 h with vigorous stirring. The mixture was then cooled to room temperature, and 10 mg of DHP was added. The dispersion was stored in a glass flask at 4 °C.

The GCE electrode (3 mm in diameter) was polished with metallographic abrasive paper (No. 6) followed by slurries of 0.3 and 0.05 μm alumina microparticles to a mirror finish. Before use, 1.0 mg DHP was added to a colloidal solution containing 1.0 mg of RGO and 1.0 mg of SDS to obtain a stable film and the RGO dispersion was sonicated in an ultrasonic bath for 30 min. 8 μL of the mixture was cast on the surface of the GC electrode, and the solvent was evaporated at room temperature for 2 h. This electrode was designated as RGO–DHP/GCE. If not used immediately, the electrode was stored at room temperature in a desiccator. The electrode preparation process is schematically illustrated in Fig. 2.

2.5. Preparation of synthetic urine

Synthetic urine was prepared using CaCl₂, NaCl, Na₂SO₄, KH₂PO₄, KCl, NH₄Cl, urea and creatinine [49]. A fixed volume of synthetic urine (900 μL fixed volume onto a microtube flask, and a 100 μL aliquot of a 1.0 × 10⁻² mol L⁻¹ estradiol solution) was carefully added and homogenized. This solution was used as a sample and it was diluted as necessary.

3. Results and discussion

3.1. GO and RGO characterization

GO was prepared by chemical exfoliation, which appeared as dark viscous solution when diluted in water. After the chemical exfoliation the treatment with ultrasonic tip and ultrasonic bath was applied to obtain a stable dispersion. Indeed, the GO obtained without treatment (A) has precipitated, and, that obtained with treatment (B) has presented a highly stable colloidal solution at different concentrations (0.05 g L⁻¹, 0.1 g L⁻¹, 0.5 g L⁻¹, and 1.0 g L⁻¹) (data not shown).
After chemical exfoliation, the presence of GO sheets was investigated using SEM. When deposited on silicon oxide (SiO₂), GO sheets generate a contrast with the substrate, enabling their visualization using optical microscopy [50–52]. For GO without treatment, a higher roughness was observed and the presence of more sheets was noted. On the other hand, treated GO presented less roughness (data not shown).

UV–Vis spectroscopy was used to verify the reduction of GO, as it has been reported in several papers [53]. An absorption band at 230 nm can be observed in GO samples (Fig. 3), which corresponds to π → π* transitions of the aromatic ring C=C bonds. A shoulder at 300 nm is also observed, which can be attributed to n → π* transitions assigned to C=O bonds. The band at 230 nm shifts to 270 nm when GO is reduced, indicating that the hexagonal lattice of GO is partially reconstituted, whereas the shoulder at 300 nm disappears, indicating the deoxygenation of GO.

It has been demonstrated that DHP can produce a stable film with CNT species [4]. It may be expected that DHP facilitates the formation of RGO composite films, increasing the exposed area of RGO. To verify the stability of RGO and GO films with DHP, ζ-potential titration was used. The magnitude and sign of the surface potential of RGO–DHP and GO–DHP sheets from pH 2.0 to 12 were determined; each point represents the average of three measurements and its corresponding standard deviation, as shown in Fig. 4. The RGO solution exhibited ζ values less than −30 mV over almost the entire range of pH values. The value increases with higher pH values, reaching a maximum of −20 mV (pH 1.0). This result is attributable to the presence of carboxylic acids in the RGO structure [54–56].

Fig. 2. Scheme of the preparation and application of RGO–DHP/GCE: 1) chemical exfoliation of graphite and treatment using ultrasonic tip and ultrasonic bath; 2) reduction of GO in presence of SDS using NaBH₄; 3) RGO–DHP dispersion preparation and casting on the GCE; and 4) voltammetric determination of estradiol.

Fig. 3. Normalized absorption spectra of GO and RGO.

Fig. 4. ζ-Potential of a colloidal solution of RGO–DHP from pH 1 to 12.
3.2. Characterization of the RGO–DHP/GCE

The electroactive area of GCE, GO–DHP/GCE and RGO–DHP/GCE was estimated in 0.1 mol L\(^{-1}\) KCl in the presence of 1.0 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) \([\text{Fe(CN)}_6]^{3-}\) according to the Randles–Sevck equation [57]:

\[ I_p = 2.69 \times 10^5 AD^{1/2}n^{3/2}ν^{1/2}c \]

where \(I_p\) is the cathodic peak current (A), \(A\) is the electroactive area (cm\(^2\)), \(D\) is the diffusion coefficient of the \([\text{Fe(CN)}_6]^{3-}\) species in solution (7.6 \(\times\) 10\(^{-6}\) cm\(^2\) s\(^{-1}\)), \(n\) is the number of electrons transferred in the redox reaction, \(ν\) is the potential scan rate (V s\(^{-1}\)), and \(c\) is the \([\text{Fe(CN)}_6]^{3-}\) concentration (mol cm\(^{-3}\)). Fig. 5a presents the cyclic voltammograms for 1.0 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) \([\text{Fe(CN)}_6]^{3-}\) in 0.1 mol L\(^{-1}\) KCl obtained with RGO–DHP/GCE, GCE and GO–DHP/GCE at 100 mV s\(^{-1}\). The electroactive areas of the GCE, GO–DHP/GCE and RGO–DHP/GCE were calculated to be 0.16, 0.30 and 0.39 cm\(^2\) respectively. Similar carbon nanotube-modified electrodes, the exposure of graphene sheets on the electrode can increase the electroactive area [4]. The RGO–DHP/GCE, GO–DHP/GCE and GCE were also characterized by electrochemical impedance spectroscopy (EIS) in the presence of 1.0 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) \([\text{Fe(CN)}_6]^{3-}\) in a 0.1 mol L\(^{-1}\) KCl solution at a potential of 0.22 V (as can be seen in Fig. 5b, which presents the Nyquist diagrams for RGO–DHP/GCE, GO–DHP/GCE and GCE). The values of electron transfer resistance depend on the dielectric properties of the electrode/electrolyte interface. We have observed small, well-defined semi-circles at higher frequencies obtained with the bare RGO–DHP/GCE, GO–DHP/GCE and GCE. As expected, the electron transfer resistance decreased dramatically upon modification of the GCE with RGO. The presence of GO on the electrode surface gave rise to a strong increase in the electron transfer resistance (30.5 kΩ) compared to that of RGO (2.73 kΩ). This increase could be because GO contains a large number of oxygen atoms, which reduces the number of C=C double bonds, decreasing the number of n bonds. In addition, the increase in electron transfer resistance can also be related with the negative charge of GO, which can repel the redox mediator. These results also demonstrated that the films containing RGO or GO were successfully immobilized on the GC surface.

3.3. Determination of estradiol using RGO–DHP/GC electrodes

Cyclic voltammograms of RGO–DHP/GCE and GCE in 0.05 mol L\(^{-1}\) PBS (pH 7.0) were recorded in the presence of 1.0 \(\times\) 10\(^{-5}\) mol L\(^{-1}\) estradiol at scan rate of 100 mV s\(^{-1}\) with accumulation time of 120 s. Both RGO–DHP/GCE (dashed line) and GCE displayed an anodic peak current related to estradiol oxidation. It was observed that RGO–DHP/GCE presented a higher anodic peak current when compared to the cyclic voltammogram obtained using bare GCE. However, two peaks, one cathodic and one anodic, near 100 mV, which were likely related to the presence of ethanol, were noted. Experiments were performed in the absence of estradiol and in the presence of ethanol, and the same peaks were observed. These peaks did not interfere with further studies. Then the electrode RGO–DHP/GCE was selected for estradiol determination. Fig. 6 shows the cyclic voltammograms of the RGO–DHP/GCE and GCE in 0.05 mol L\(^{-1}\) PBS (pH 7.0) and 1.0 \(\times\) 10\(^{-5}\) mol L\(^{-1}\) estradiol at a scan rate of 100 mV s\(^{-1}\), with accumulation time of 120 s. The effect of scan rate on the voltammetric response of the RGO–DHP/GCE in 0.05 mol L\(^{-1}\) PBS (pH 7.0) in the presence of 1.0 \(\times\) 10\(^{-5}\) mol L\(^{-1}\) estradiol was evaluated. The anodic peak currents were linear from 10 to 150 mV s\(^{-1}\) (the linear regression equation was \(I_p = 8.0 \times 10^{-6} + 3.3 \times 10^{-4} ν\) (\(R = 0.994\)), suggesting that the electrochemical oxidation of estradiol is controlled by adsorption. The \(E_p\) of the oxidation peaks was also dependent on scan rate; which can be analyzed using Laviron’s equation [58]:

\[ E_p = E_0 + \frac{RT}{qF} \ln \frac{RTk_c}{qF} - \frac{RTk}{qF} \]

![Fig. 5. (a) Cyclic voltammograms obtained with RGO–DHP/GCE (dashed line), GO–DHP/GCE (solid line) and GCE (inset) electrodes at 100 mV s\(^{-1}\) in the presence of 1.0 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) \([\text{Fe(CN)}_6]^{3-}\) in a 0.1 mol L\(^{-1}\) KCl. (b) Nyquist diagrams of RGO–DHP/GCE (●), GO–DHP/GCE (▲) and GCE (■) in the presence of 1.0 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) \([\text{Fe(CN)}_6]^{3-}\) in a 0.1 mol L\(^{-1}\) KCl solution at a potential of 0.22 V. RGO–DHP/GCE (●) and GCE (■) Nyquist diagrams (inset).](image)

![Fig. 6. CVs of RGO–DHP/GCE (dashed line) in 0.05 mol L\(^{-1}\) PBS (pH 7.0) in absence and presence of 1.0 \(\times\) 10\(^{-5}\) mol L\(^{-1}\) estradiol at scan rate of 100 mV s\(^{-1}\), with accumulation time of 120 s. CVs of GCE (inset) (solid line) in 0.05 mol L\(^{-1}\) PBS (pH 7.0) in absence and presence of 1.0 \(\times\) 10\(^{-5}\) mol L\(^{-1}\) estradiol at scan rate of 100 mV s\(^{-1}\), with accumulation time of 120 s.](image)
where $E_p$ is the anodic peak potential, $\alpha$ is the transfer coefficient, $v$ is the potential scan rate ($V \, s^{-1}$), $k_i$ is the heterogeneous electron transfer rate constant ($s^{-1}$), $n$ is the number of electrons for estradiol oxidation [42] and $R$ and $T$ have their usual meanings.

According to Bard and Faulkner [57], $\alpha$ can be given as

$$\alpha = \frac{47.7}{E_p - E_p/2}$$

where $E_p/2$ is the potential, which the current is at half the peak value. From this equation, we obtained a value of $\alpha = 0.54$. The linear regression equation of the plot of $E_p$ versus $\ln v$ was $E_p = 0.73 + 0.052 \ln v$, with a correlation coefficient of 0.994. The estimated value for $k_i$ was 0.14 s$^{-1}$.

We have investigated the influence of accumulation time on the oxidation peak current of $1.0 \times 10^{-6}$ mol L$^{-1}$ estradiol from 0 to 270 s (Fig. 7). The best definition and maximum peak current were obtained at an accumulation time of 240 s. After this time, the signal started to level off, which could be attributed to the fact that the amount of estradiol on the modified electrode surface had greatly increased. Therefore an accumulation time of 240 s was selected for further studies.

The relationship between the increase in the oxidation peak current and the concentration of estradiol was evaluated. Under optimized conditions, the peak current increased proportionally with the estradiol concentration in the range from $4.0 \times 10^{-7}$ to $1.0 \times 10^{-5}$ mol L$^{-1}$; with the linear regression equation $\Delta I_{pa} (\mu A) = 1.65 + 3.54 \times 10^6$ [estradiol] (mol L$^{-1}$), and a correlation coefficient of 0.995 (Fig. 8). The detection limit was $7.7 \times 10^{-8}$ mol L$^{-1}$ (obtained by using three times the standard deviation of the signal blank/slope of the calibration curve).

Intra-day and inter-day repeatability ($n = 5$) tests were carried out with one sensor in presence of $1.0 \times 10^{-6}$ mol L$^{-1}$ estradiol. The RGO-DHP/GCE exhibited a relative standard deviation of 2.7% and 3.8% for intra-day and inter-day repeatability, respectively. On the other hand, a relative standard deviation of 6.4% was obtained for measurements of $1.0 \times 10^{-6}$ mol L$^{-1}$ estradiol using five different electrodes prepared in the same way on 5 different days (repeatability study). Finally, the stability of the sensor was also investigated by measuring its response in $1.0 \times 10^{-6}$ mol L$^{-1}$ estradiol during 15 days; and, in the end of this period the response current decreased only 10%.

### 3.4. Interference studies and sample analysis

The performance of the RGO–DHP/GCE electrode toward estradiol detection was evaluated in the presence of common interferent compounds present in human urine. Each of the following compounds: CaCl$_2$, NaCl, Na$_2$SO$_4$, KH$_2$PO$_4$, KCl, NH$_4$Cl, urea, creatinine, fructose, D-galactose, L-tyrosine, L-histidine, glycine and glucose (all in 100-fold excess) was individually added to a $1.0 \times 10^{-6}$ mol L$^{-1}$ estradiol solution in 0.05 mol L$^{-1}$ PBS (pH 7.0). The influence of ascorbic acid using the same concentration of estradiol ($1.0 \times 10^{-6}$ mol L$^{-1}$) was also investigated. Using linear sweep adsorptive stripping voltammetry technique, these chemical species caused no significant interference (less than 5%) in the electrode response current and did not affect the determination of estradiol using the RGO–DHP/GCE. Additionally, the RGO–DHP/GCE was applied to the determination of estradiol in synthetic human urine samples by standard addition, and the recovery value for several estradiol concentrations, namely A, B and C ranged from 94 to 101%, as it may be seen on Table 1.

The characteristics of the proposed electrode were compared with those reported in the literature to the same analyte (Table 2). It can be noted that the RGO–DHP/GCE presented similar linear range and limit of detection as compared to most of the other electrochemical sensors developed to determine estradiol. Indeed, we can highlight that the electrode shows good performance in the determination of estradiol using the voltammetric analysis.

### 4. Conclusions

A novel treatment to obtain GO was developed using ultrasonic tip and ultrasonic bath, which proved to be an efficient method to maintain stability dispersion of GO. DHP was used in conjunction with RGO and promoted the formation of a stable dispersion, which was cast on a GCE and used in the determination of estradiol.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (10$^{-5}$ mol L$^{-1}$)</th>
<th>Proposed electrode (10$^{-5}$ mol L$^{-1}$)</th>
<th>Recovery (%)</th>
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<tr>
<td>A</td>
<td>4.00</td>
<td>4.03</td>
<td>101</td>
</tr>
<tr>
<td>B</td>
<td>16.0</td>
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<td>98.8</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>94</td>
<td>94</td>
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</table>
A linear response was obtained over a concentration range from 4.0 × 10^{-7} to 1.0 × 10^{-3} mol L^{-1}, with a detection limit of 7.7 × 10^{-9} mol L^{-1}. which are good results as compared with those reported in the literature. RGO–DHP is a composite material that can be easily prepared and has been proven to be a selective electrode for estradiol analysis in synthetic urine samples using linear sweep adsorptive stripping voltammetry. Therefore, we can suggest that films containing RGO and DHP are attractive for applications as electrochemical sensors and biosensors.

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