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Voltammetric techniques for low-cost on-site routine analysis of thymol in the medicinal plant *Ocimum Gratissimum*.

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Keywords: electrochemistry, essential oils, *Ocimum gratissimum*, thymol, chemical composition variation.

Highlights

- Electrochemistry allows titration of thymol + carvacrol content in essential oils of *Ocimum gratissimum*
- Measurements can be performed directly in an infusion
- A low cost and portable electrochemical set-up is proposed

Abbreviations

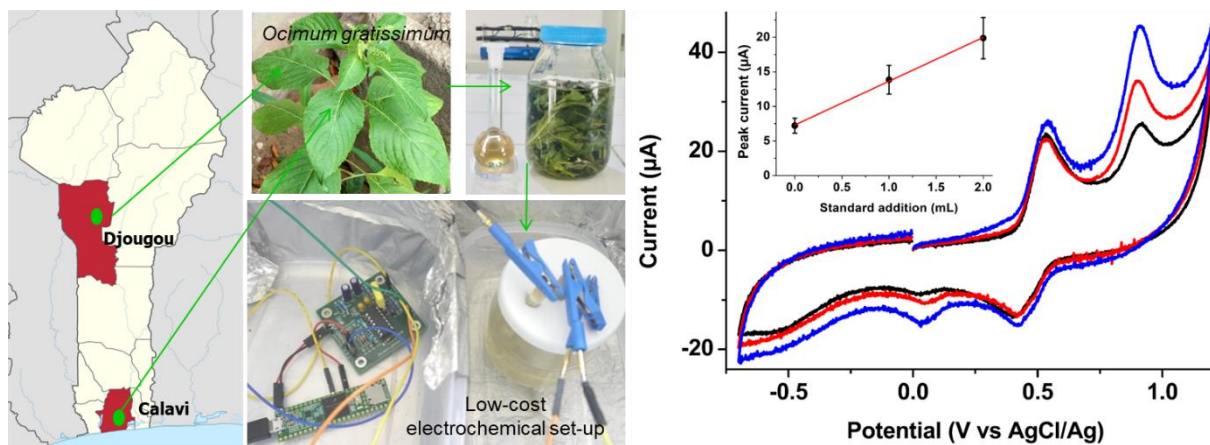
CV: cyclic voltammetry

OG: *Ocimum gratissimum*

GC: glassy carbon

SWV: square wave voltammetry

Graphical abstract



A low-cost electrochemical set-up is used to evaluate the thymol concentration in essential oils and infusions *Ocimum gratissimum*, a medicinal plant from Benin.

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Abstract

The composition of essential oils varies according to culture conditions and climate, which induces a need for simple and inexpensive characterization methods close to the place of extraction. This appears particularly important for developing countries. Herein, we develop an analytical strategy to determine the thymol content in *Ocimum Gratissimum*, a medicinal plant from Benin. The protocol is based on electrochemical techniques (cyclic and square wave voltammetry) implemented with a low cost potentiostat. Thymol is a phenol derivative and was directly oxidized at the electrode surface. We had to resort to submillimolar concentrations (25-300 μM) in order to minimize production of phenol oligomers that passivate the electrode. We worked first on two essential oils and realized that in one of them the thymol concentration was below our detection method. These results were confirmed by gas chromatography – mass spectrometry. Furthermore, we optimized the detection protocol to analyze an infusion made directly from the leaves of the plant. Finally, we studied whether the cost of the electrochemical cell may also be minimized by using pencil lead as working and counter electrodes.

Introduction

Alternative treatments compared to occidental pharmaceuticals often rely on the use of essential oils [1]. These are important in developing countries where traditional medicine is still the main source of health care and for which access to treatments at reasonable cost may be difficult as reported by the World Health Organization [2]. A key issue for herbal health is the quality of the product in terms of chemical composition, safety and efficacy. Quality control of herbal health products is a critical challenge for its promotion and integration into health systems. One of the main difficulties is the variation in the chemical composition of plants due to genetic, agricultural and environmental factors (seasonal and climatic variations, soil composition), as well as in the process of collection, drying, storage and extraction that further influence the content of plant materials [3–6]. Therefore, ideally, the amount of active components present in an herbal health product such as herbal tea or essential oil should be indicated and should be above a certain efficacy threshold. Typically, mass spectrometry approaches are used to decipher composition variations. Those provide excellent quantification and exhaustivity of all products [7–9]. Nevertheless, this heavy and expensive instrumentation requires specific training to be used properly. As a consequence, their use is restricted to specific research purposes and limited to a few samples per system. Hence, there is an avenue to propose alternative ways of quantifying the active molecules in those complex mixtures. More specifically, many plants contain antioxidants that are useful to fight oxidative stress and reduce the risk of many non-communicable diseases such as cardiovascular pathologies, cancers, atherosclerosis, diabetes, Alzheimer's disease, cataracts, and other respiratory disorders [10,11]. Therefore, those systems are often possibly oxidized at an electrode surface, even in aqueous media.

While the most accurate methods for analyzing those complex media remain mass spectrometry [12], access to lightweight and low-cost instruments that can be directly deployed on-site would be of great interest. In this area, electrochemical methods appear very attractive and adapted [13]. Indeed, potentiostats can be miniaturized and easily transported and powered by batteries (often from a laptop) [14–17]. Sophisticated electrode preparation procedures may be necessary if sub- μM concentrations of organic molecules need to be detected, but conventional electrodes are easily sensitive to concentrations in the sub-millimolar range without modification [18]. In addition, very cheap potentiostat configurations are nowadays proposed through open access instrumentation [16].

Below, we will explore how low-cost electrochemical analysis can be used to monitor the concentration of an active ingredient in an essential oil and an infusion. We choose the example of *Ocimum gratissimum* (OG) that grows and is frequently consumed in several parts of Benin. The leaves and roots are widely used traditionally in cooking, in medicinal recipes against fungal, urinary, infections, gonorrhoea, bronchitis, vertigo, diarrhoea, vomiting, respiratory, cardiovascular and liver diseases, fever and malaria, and as an insecticide [19–23]. OG contains thymol, a phenol derivative that can be oxidized chemically or electrochemically and is biologically active [24–29]. In order to simplify the analytical procedure, we used only unmodified electrodes. Since thymol produces oligomers upon oxidation, we

first examined the thymol electrochemical behavior in a first electrolyte (acidic conditions) to evaluate the optimal concentration range where sensitivity is sufficient but electrode passivation, that induces a departure from linearity, avoided. Then, quantification of thymol in two OG essential oils was performed and compared with independent mass spectrometry and IR measurements. Subsequently, the conditions were optimized to carry out direct measurements in an aqueous infusion made directly from the plant leaves. In order to propose a full electrochemical setup at a reduced cost, in a final study we considered the use of pencil leads to perform the detection.

Material and methods

Electrochemical experiments were performed at the Laboratory of Biochemistry and Bioactive Natural Substances, Faculté des Sciences et Technique of Université d'Abomey-Calavi. Therefore, experimental procedures were adapted to the local conditions.

Products. Sulfuric acid (VWR, 95-98%), Citric acid (Aldrich, 99.5%), Sodium chloride (VWR, 99.9%), absolute ethanol (Aldrich, > 99.8%) and Thymol (Aldrich, 98.5%) were used as received. We realized that distilled water was not pure enough so that we resorted to the local mineral water Fifa whose composition is provided in supporting information. One advantage is that mineral water only contains electroinactive ions and that its composition is verified and public anywhere on the planet. Electrolyte was either a 50/50 volumetric mixture of 0.5 M sulfuric acid and ethanol, or an aqueous solution containing 0.1 M NaCl and 0.1 M citric acid.

The leaves of OG were collected in their natural habitat in the township of Abomey-Calavi, department of the Atlantic, agroecological zone n°6, southern Benin for sample 1 and in the township of Djougou, agroecological zone n°4, northern Benin for sample 2. For essential oil extraction, the leaves were first dried for several days. Then, 100 g of collected material in each township and 500 mL of distilled water were introduced in 1 L flask and fixed onto the hydrodistillation Clevenger apparatus system. Each extraction was performed continuously for 2-3 hours. The essential oil was collected into an amber bottle protected from light by aluminum foil. For the infusion, the fresh leaves of OG were collected in an organic garden in the township of Sèmè-Podji, agroecological zone n°8, southern Benin. Infusions were prepared by adding 250 mL of boiling mineral water to 25 g of OG leaves. We observed that it is important to close the recipient otherwise thymol can escape with vapours.

Potentiostat and electrochemical cell. We resorted to the open source potentiostat PassStat 2.1 recently reported by some of us, and which costs ca 50€ [16]. Briefly, this potentiostat is powered and controlled by the USB port of a laptop. In Benin, the 50 Hz electrical grid is not always stable and contains many parasites, which introduces significant noise into the measurements. To face that issue, we carried out the measurements without connecting the laptop to the mains. In addition, the potentiostat and electrochemical cell were placed in a home-made Faraday cage made with aluminum foil connected to the ground of the potentiostat. The current/voltage conversion resistance R_6 of the potentiostat was

manually adjusted to fit the measured current, as well as the capacitance CF_3 that filters the noise. Taking the same notations as in our previous work [16], typical values for a 2 mm diameter working electrode are $R_6 = 100 \text{ k}\Omega$, and $CF_3 = 10 \text{ nF}$. The frugal electrochemical cell was made in a glass yogurt container with holes in the cap to insert the electrodes. No inert gas was available so that experiments were performed under atmospheric environment and at room temperature ($27 \pm 3^\circ\text{C}$). Pictures of the set-up are provided in Figure 1. Oxygen reduction wave was used to assess that the electrode was not passivated. We alternatively used an Emstat Pico from Palmsens to compare our results with a commercial system. No noticeable difference was found.

OG solutions were prepared by adding $100 \mu\text{L}$ of OG essential in a 10 mL volumeter flask that was then filled with pure ethanol. Then, aliquots were added to a 50 mL electrolyte solution. For calibration, a 25 mM solution of thymol in the electrolyte was prepared.

Electrodes. Working electrodes were either a 2 mm diameter glassy carbon electrode from Orignalys or a 3.15 mm diameter 6B pencil lead from Faber Castell. Electrodes were hand polished with P4000 paper and then $0.3 \mu\text{m}$ alumina excepted for the pencil lead for which only P4000 was used. Then, the electrode was sonicated for 2 min in pure mineral water. We observed an important dependence of the voltammogram shape and peak currents on the surface state of the electrode. Three assays were performed by sample and two groups performed independent calibration and measurements with different potentiostats and electrodes, but identical OG oil and electrolytes. Thus, electrodes were polished after each voltammogram, and datas presenting obvious fouling of the electrode even after polishing were discarded. A baseline subtraction was performed to evaluate the faradaic peak currents. Counter electrode was a 0.5 mm diameter platinum wire or a pencil lead. Reference electrode was either a commercial (CHI) or a home-made AgCl/Ag reference electrode.

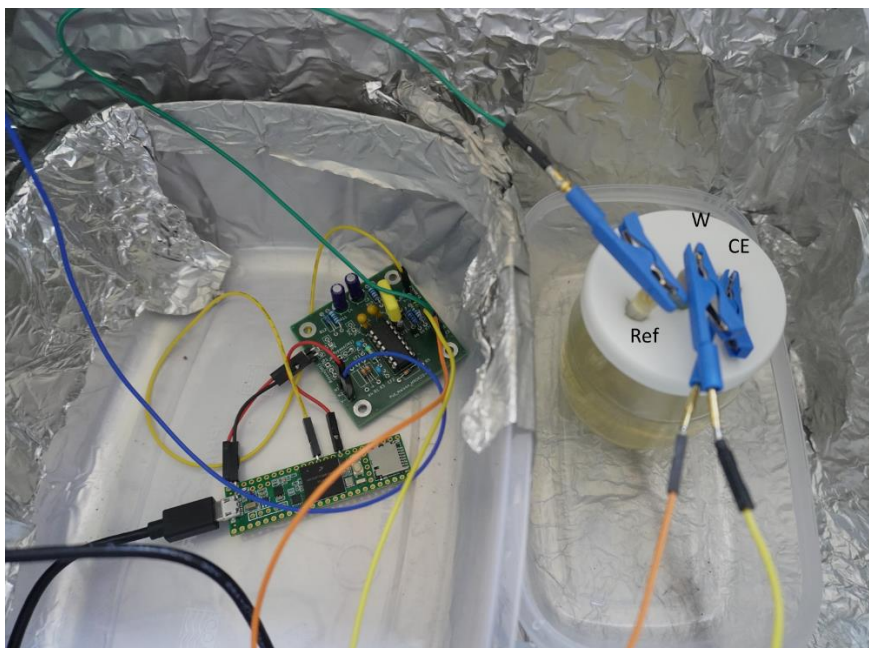


Figure 1. Picture of the low-cost electrochemical setup.

Mass spectrometry

Gas Chromatography – Mass Spectrometry was performed on an Agilent 5977A-7890B system with electronic impact. Acquisitions were performed in the single ion monitoring mode. The capillary GC column was a HP-5MS.

Results and discussion

1. *Thymol electrochemical reactivity*

Since the extraction of essential oils is made by hydrodistillation, the products obtained are often very poorly soluble in water. Therefore, we first used a 50/50 volumetric mixture of 0.5 M sulfuric acid/ethanol to prepare our electrolyte. Acidic solutions were chosen because the electrooxidation peaks of phenol derivatives are larger and well-defined under these conditions. Figure 2 presents the cyclic voltammogram obtained for a 149 μM of thymol on a glassy carbon electrode at 0.1 V s^{-1} . A chemically irreversible peak is observed at $0.97 \text{ V vs AgCl/Ag}$. On the backward scan, a small wave is detected at $0.12 \text{ V vs AgCl/Ag}$. This wave is chemically reversible as observed on the second scan which presents a peak at $0.49 \text{ V vs AgCl/Ag}$. The precise nature of the products formed is unknown, but it is well-known that thymol and other phenol derivatives produce oligomers or polymers that are easier to oxidize upon a 1 electron 1 proton oxidation [27,28,30]. These polyphenolic systems are logically easier to oxidize than their parent thymol. As a consequence of electrode passivation, at the second and successive cycles, the peak shape is smoothed and the current diminished. The inset of Figure 2 shows the corrected peak current (subtracted from a linear baseline) of the first cycle as a function of thymol concentration. It is observed that above $200 \mu\text{M}$, the linearity is lost because electropolymerization and electrode

passivation are already effective, resulting in a peak potential shift and lower peak current. Within a concentration range between 50 and 200 μM , we estimate that the peak current allows to estimate the thymol concentration with a 10% error. A precise evaluation of the detection threshold was however not performed since in our case we handle rather large concentrations. In the literature, it is well-established that carvacrol, a regioisomer of thymol, presents similar electrochemical characteristics. Nevertheless, since it has been shown that carvacrol is only a minor product in the oil produced in Benin, we did not consider a full electrochemical characterization of this compound [8,9].

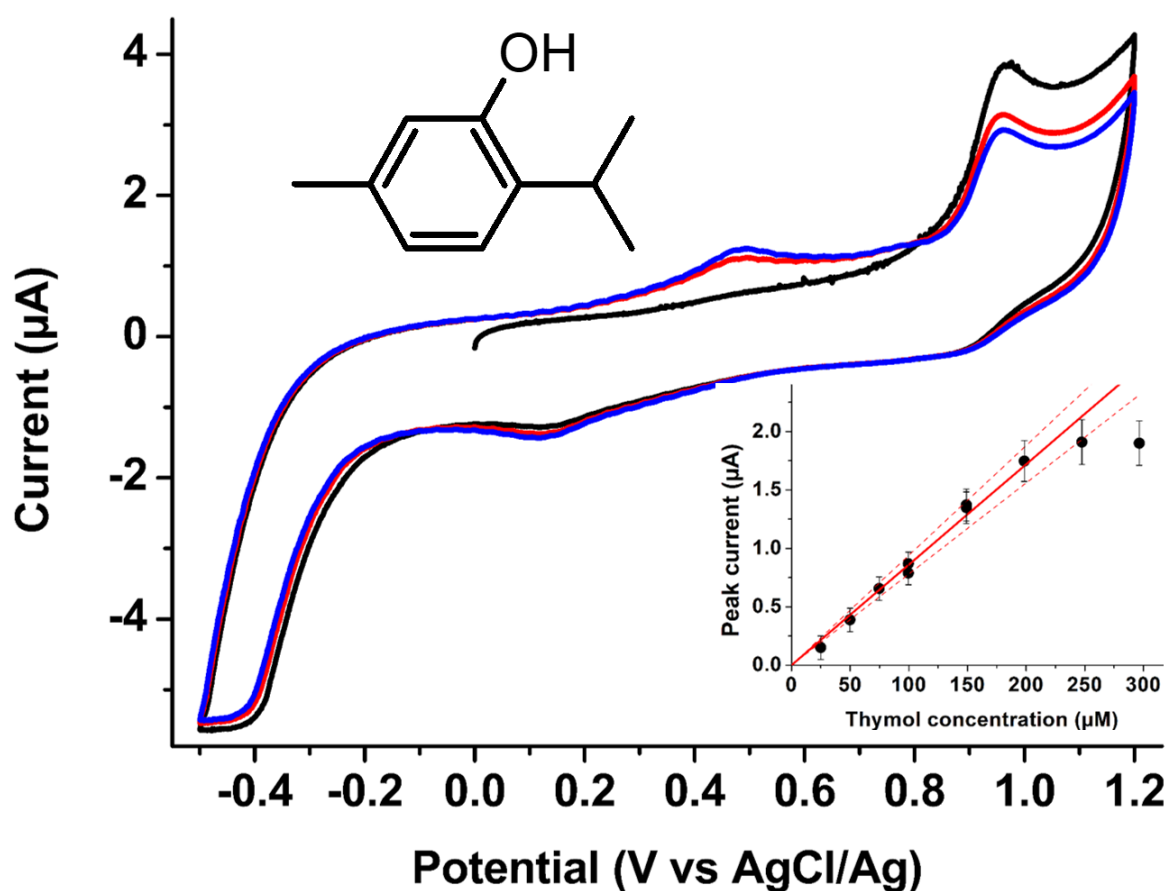


Figure 2. Cyclic voltammogram of glassy carbon electrode in 50/50 0.5 M sulfuric acid/ethanol solution containing 149 μM thymol recorded with a commercial potentiostat at 0.1 V s^{-1} . Inset: evolution of the peak current of the first cycle with thymol concentration c (black dots). $I(\mu\text{A}) = 0.043c(\mu\text{M})$.

Since electrode passivation is strongly dependent on the initial solution composition of the solution, but also on the surface state and thus on the polishing, it seemed risky to work beyond the linear range even if one study reports electropolymerization to discriminate between thymol and carvacrol that present oxidation at identical potentials [31]. Therefore, in the next section, we diluted the OG oil in order to stay in the appropriate concentration range and similarly checked the linearity range of the electrochemical response.

2. Quantification of thymol content in OG essential oils

The chemical composition of medicinal plants and more specifically OG can vary depending on several factors such as the climate, area and time of harvest, soil parameters and phenology of plants [8,9]. For example, the essential oil of OG leaves harvested in Brazil is rich in Eugenol and Eucalyptol [32] while those harvested in Benin are rich in Thymol, p-cymene and γ -terpinene [33]. In Benin, Kpadonou et al. showed that two chemotypes have been observed depending on the time of harvest: p-cymene/thymol chemotype for plants harvested at 7 a.m. and p-cymene chemotype for those harvested at 1 p.m. or 7 p.m. [9]. In the present study, leaves of *O. gratissimum* were harvested in two agro-ecological areas which differ in climate, soil type and rainfall. We collected one sample from the township of Abomey-Calavi (sample 1, southern Benin) and one from the township of Djogou (sample 2, northern Benin) to illustrate the geographic variability and the potential of electrochemistry as a simple discrimination method. Each oil was diluted 100 times in ethanol and aliquots were added to 50 mL of electrolyte. CVs were then acquired as in the previous section. Figure 3a shows the results obtained for OG-sample 1. It is clear that the curves exhibit similarities with those presented in the previous section, which suggests that their shape is determined by the thymol content of the oil. As in the previous section, we checked the linearity of the response for sample 1 and determined that below 400 μ L the peak current could be used to determine the OG concentration. The average of all measurements yielded a thymol concentration of 25 ± 5 mM in the diluted sample 1, therefore 2.5 ± 0.5 M in the pure oil. Reproducibility was checked and another operator found 2.7 ± 0.5 M. Thus, the accuracy is highly sufficient to determine the thymol concentration in OG essential oil.

Figure 3b presents the cyclic voltammograms after the addition of 200 μ L of OG-sample1 or 1400 μ L of OG-sample 2. Although a clear signal is observed for sample 1, nothing is visible for sample 2.

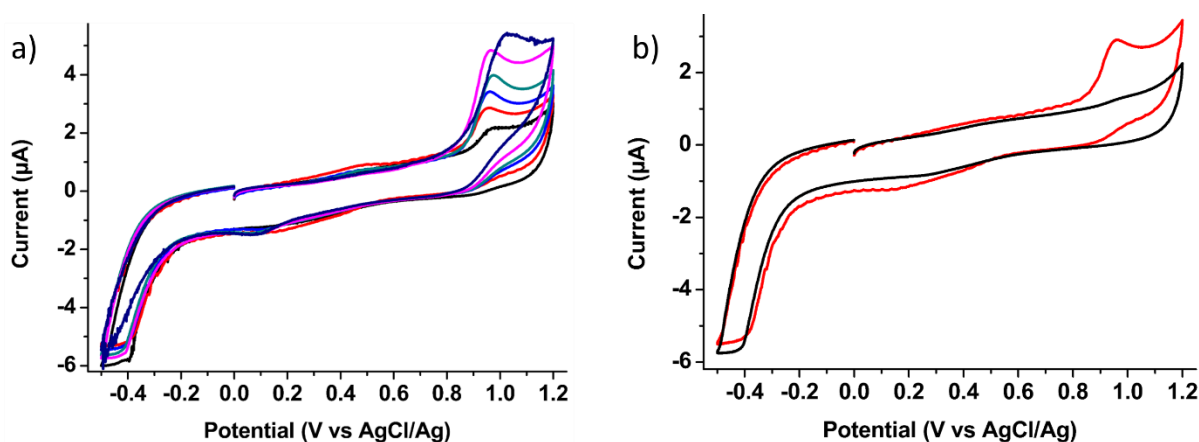


Figure 3. Cyclic voltammograms obtained on a glassy carbon electrode after different additions of OG-sample 1 and OG-sample 2, previously diluted 100 times in ethanol, to 50 mL of electrolyte. Data were recorded with the open source potentiostat PassStat 2.1 at 0.1 V s^{-1} : a) CV of OG-sample 1 after addition

of 100 (black), 200 (red), 300 (blue), 400 (green), 500 (pink) and 700 (navy) μL in supporting electrolyte; b) CV of OG-sample 1 after addition of 200 μL (Calavi, red) or of 1400 μL of OG-sample 2 (Djogou, black) in supporting electrolyte.

In order to get further information on this striking difference between the two samples, a quantification of thymol and carvacrol content in our samples was performed by gas chromatography – mass spectrometry. We found 2.34 M for sample 1 (2.20 M thymol + 0.15 M carvacrol) and 6.2×10^{-3} M (3.1×10^{-3} thymol and 3.1×10^{-3} M carvacrol) for sample 2, as detailed in Supporting Information, in very good agreement with our electrochemical measurements. These first results demonstrate that cyclic voltammetry and classical electrochemical setups without extensive sample or electrode preparation are already sufficient to discriminate between plant extracts that contain significant amounts of thymol or not.

3. Electrochemistry within an infusion

After obtaining the probing results mentioned above, we decided to shift our focus to the analysis of an infusion. After preparing the infusion, we added 0.1 M citric acid + 0.1 M NaCl as electrolyte, as these inexpensive products can be easily obtained. We observed with this new electrolyte a positive effect, *i.e.* electrode fouling by oligomers was reduced under these conditions, extending the linearity range to higher concentrations. This could be due to hydrogen bonding between OH groups of thymol derivatives and citric acid. However, we had to dilute the initial solution five times with pure electrolyte to stay in the linear range. Figure 4a presents the CV obtained for the system. A large chemically reversible peak is observed at 0.554 V *vs* AgCl/Ag. This peak is induced by the soluble antioxidant species such as flavonoids, tannins, phenolic compounds or glycosides [26,34,35]. The second small peak is attributed to thymol, as previously identified. In this study, we also resorted to square wave voltammetry (SWV, see Figure 4b), that is known to be more sensitive and can better distinguish species when peaks overlap [13]. We tested here the sample addition method and observed a linear behavior up to the addition of 3 mL of 25 mM thymol into 50 mL of electrolyte. SWV yielded a thymol concentration of $550 \pm 85 \mu\text{M}$ in the final solution (thus $2.8 \pm 0.5 \text{ mM}$ in the initial one).

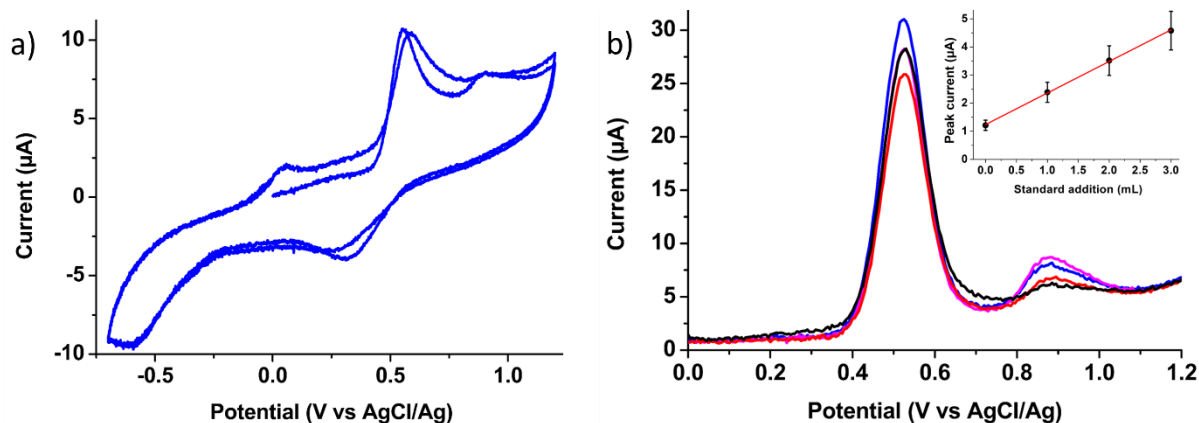


Figure 4. a) Cyclic voltammogram of a glassy carbon electrode recorded in OG infusion + 0.1 M citric acid + 0.1 M NaCl solution recorded with an Open source PassStat 2.1 potentiostat at 0.1 Vs^{-1} ; b) Square wave voltammograms of glassy carbon electrode in electrolyte solution (50 mL) + addition of 0 mL (black), 1 mL (red), 2 mL (blue) and 3 mL (pink) of 25 mM thymol standards; Inset: peak current I vs standard addition volume V . $I(\mu\text{A}) = 1.24 + 1.12V(\text{mL})$.

4. Low-cost electrochemical cell

In a final attempt to minimize all costs, we replaced the glassy carbon working electrode and the platinum counter electrode with pencil leads. In fact, pencil leads are made of graphite and a polymer to ensure cohesion [36]. In the literature, such electrodes are emerging for several electroanalytical purposes [37], and have been used for the evaluation of antioxidants in tea [38], for the titration of eugenol (another phenol derivative) [39] and for the determination of copper in sea water after electrode modification with a specific complexing molecule [40] among other examples. Different ratios graphite/polymer are available, and we chose 3.15 mm diameter 6B pencil leads insulated with epoxy resin since they present a high graphite load but are still mechanically resistant. Figure 5 presents three CVs obtained after the introduction of a freshly polished electrode in the precedent solution, and the results of the current evolution for the sample addition method (1 and 2 mL were added to 50 mL of solution). We determined a concentration of $580 \pm 85 \mu\text{M}$ of thymol with the pencil lead ($2.9 \pm 0.5 \text{ mM}$ in the initial infusion) for the same solution than in the previous section. Nevertheless, we have to point out that we have also noticed that the thymol wave increases with time whereas the one of antioxidants remains rather constant so that we present here results obtained after keeping the electrode 5 min in the solution prior starting the voltammetric acquisition. This indicates that for these electrodes, thymol adsorption contributes to the current which can then be larger than expected. In fact, adsorption results in higher current density, which may be an advantage and be exploited in the future to detect lower concentrations. However, it is also a source of irreproducibility. Further work will be performed in the future to propose an accurate methodology for complex mixtures containing systems that adsorb to the pencil lead electrodes, possibly by introducing a pre-anodization of the electrode [41]. Otherwise, using

a cylindrical pencil lead electrode as a counter electrode was not problematic and even provided a larger counter electrode. Since we used a home-made AgCl/Ag reference electrode, the total price of the electrochemical cell is less than 5 €.

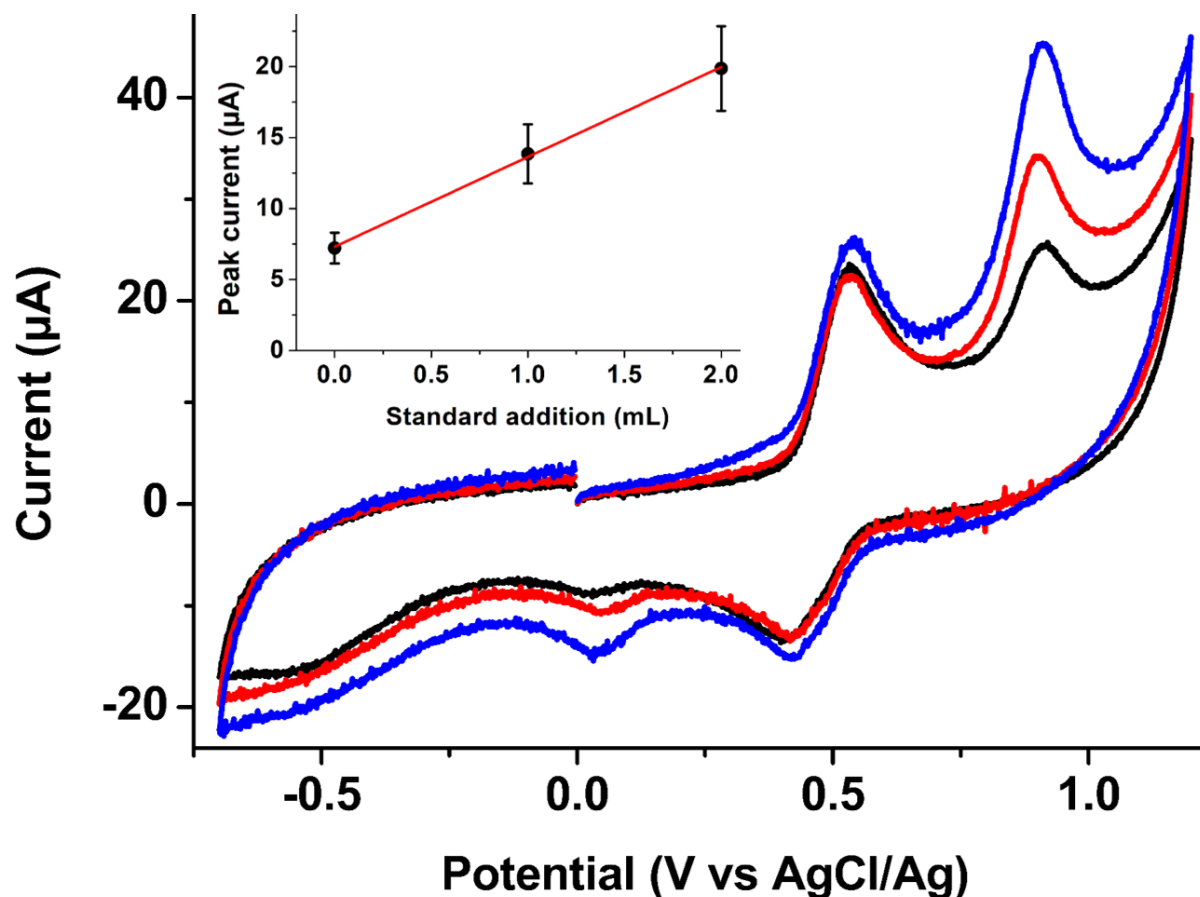


Figure 5. Cyclic voltammograms of a 3.15 mm diameter 6B pencil lead electrode recorded in OG infusion + 0.1 M citric acid + 0.1 M NaCl electrolyte recorded with an Open source PassStat 2.1 potentiostat at 0.1 V s^{-1} after addition of 0 mL (black), 1 mL (red) or 2 mL (blue) of 5 mM thymol standard to 50 mL of electrolyte. Inset: peak current I vs standard addition volume V . $I(\mu\text{A}) = 7.31 + 6.33V(\text{mL})$.

Conclusion

Overall, our work illustrates how analytical electrochemistry can be implemented in non-ideal experimental environment. The electrochemistry of thymol is challenging due to oligomerization, which partially blocks the electrode surface and requires the experimentalist to polish the electrode after each voltammogram. Nevertheless, we have successfully determined that peak current proportionality with concentration is valid in micromolar solutions, enabling quantification in a sulfuric acid/ethanol mixture

that can be probably of interest for quantification of other substances poorly soluble in water. We could easily ascertain that OG sample from Abomey-Calavi contains a rather large quantity of thymol whereas none was detected in the sample from Djougou. This is an important finding in view of future biological activity tests. Furthermore, using only water but citric acid and sodium chloride as electrolyte, thus cheap and worldwide available compounds was found to provide excellent quantification of thymol in infusions. These measurements were performed with a low-cost electrochemical apparatus. Pencil lead electrodes and home-made reference electrodes may also be used to minimize the cost. Future improvements may involve optimization of electrode preparation or adjusting the voltammetric scan rate. A slight increase in the scan rate may prevent oligomerization while keeping a low capacitive background that is typical for carbon electrode and without introducing limitations due to electron transfer kinetics. This study demonstrates that electrochemistry may be implemented at a low cost for both teaching and research purposes, while still keeping performances close to those shown by commercial systems. More particularly, this technique is highly relevant for the determination of concentrations of active molecules in complex mixtures such as essential oils or herbal teas, for the further development of quality control of plant-based health products.

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