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Electrochemical Genosensors as a New Approach on Plant DNA Detection and Quantification for Honey Authentication †

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Abstract: Honey is a natural sweet food product with multiple nutritional and medicinal properties making it a healthy alternative to processed sugars. With the consumers' recent interest and purchase of dietary products the global honey market has greatly increased. To keep up with production, or simply for financial gain, some producers/companies are now blending pure honey with cheaper substances that possess similar physical characteristics. As there are no notable visible differences between the pure and adulterated honey, it is extremely difficult to determine the purity of the available honeys. In this study, an electrochemical genosensor based on the sandwich format DNA hybridization reaction between two complementary probes was developed for the detection and quantification of *Erica arborea* pollen DNA in real samples. Analyzing public database platforms, a 98 base-pair DNA-target probe capable of unequivocally detecting the pollen from *E. arborea* was selected and designed. The complementary probe to the DNA-target oligonucleotide sequence was then cut into a 28 base-pair thiolated DNA-capture probe and a 70 base-pair fluorescein isothiocyanate-labelled DNA-signaling probe. To increase the hybridization reaction, a self-assembled monolayer formed from mixing the DNA-capture probe with mercaptohexanol was employed. Using chronoamperometry, the enzymatic amplification of the electrochemical signal was achieved with a concentration range of 0.03 to 2.00 nM. The DNA from certified *E. arborea* leaves was extracted using liquid nitrogen and mechanical grinding and the targeted region amplified by PCR. The developed genosensor was successfully applied for the detection and quantification of the DNA concentration of the extracted *E. arborea* plant leaves. So, the developed genosensor is a promising cost-effective and innovative analytical method to detect and quantify the DNA concentration of plant DNA in real honey samples.

Keywords: botanical origin; electrochemical genosensor; *Erica arborea*; honey authentication; molecular biology

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1. Introduction

Food fraud is a growing concern for the food industry [1]. This fraudulent practice occurs when food producers and/or suppliers intentionally deceive their customers about the quality and/or composition of the food they distribute [2]. It is predicted that food fraud affects the global food industry by approximately 30 billion euros annually [2,3]. In the European Union, honey is among the most adulterated products found in the market, with a revenue loss of about 600 million euros worldwide [2].

As a natural sweet food with a rich nutritional composition and multiple health benefits (e.g., anti-inflammatory, antioxidant, and antimicrobial properties), honey is often consumed as a healthy alternative to processed sugars [4]. Nevertheless, its nutritional composition differs based on the climate, soil, altitude, production method, and pollen source, consequently affecting its health benefits and market value [5]. So, the price of honey will differ significantly depending on its botanical and geographical origins since different origins affect the quality, flavor, and/or health benefits that they exhibit [6]. This makes honey vulnerable to adulteration [7,8].

To keep up with consumers' demand, or simply for monetary gain, some producers resort to fraudulent acts such as the adulteration of high-quality honey with lower-quality substances and the mislabeling of its origin and nutritional profile, compromising the safety and quality of honey [7–9]. Therefore, safeguarding the consumers' interests and promoting the sustainable growth of the food industry hinges on combating food fraud. Hence, food authenticity is an important field in food safety and quality control, especially amidst the expanding global market and intricate agri-food production systems [6,9].

Several techniques have been employed (e.g., stable carbon isotope ratio analysis, gas and liquid chromatography, nuclear magnetic resonance spectroscopy, infrared spectroscopy, etc.), nonetheless, honey authentication is a complex process [10]. Normally, melissopalynology is employed to identify the botanical provenance of honey and to learn more about its geographical origins. However, due to the variety of certain plant species' pollen morphology, this assay is time-consuming and requires skilled professionals with substantial expertise [10,11].

In this study, an electrochemical genosensor capable of detecting *Erica arborea* (white heather flower) pollen DNA with high sensibility and selectiveness was developed. A sandwich hybridization format was chosen to enhance the sensor's selectivity and avoid the formation of secondary structures. Therefore, the sensor's methodology consisted of a sandwich hybridization between a complementary 28-mer DNA sequence (designated as DNA-capture probe), attached to the surface of a screen-printed gold electrode (SPGE), and a 98-mer *E. arborea* oligonucleotide sequence. For a complete hybridization, another complementary 70-mer DNA sequence (designated as DNA-signaling probe) to the white heather flower was designed using a fluorescein isothiocyanate (FITC) to which anti-fluorescein antibodies labelled with horseradish peroxidase (POD) enzymes were attached. The enzymatic amplification of the analytical signal was obtained by chronoamperometry using a POD/H_2O_2 system. A linear relationship between electrochemical intensity and DNA concentration was observed when DNA concentrations ranged from 0.03 to 2.00 nM. The developed sensor was applied to the detection of the DNA from real *E. arborea* plant samples with promising results. This sensor will hopefully determine the geographic botanic origin of honeys and facilitate honey food safety and control.

2. Materials and Methods

2.1. Reagents and Solutions

3,3′,5,5′ tetramethylbenzidine (TMB), 6- mercapto-1-hexanol (MCH) and 20× sodium phosphate-EDTA (200 mM sodium phosphate, 3 M NaCl, 20 mM EDTA) solution (20× SSPE) were attained from Sigma Aldrich (Mannheim, Germany), the phosphate-buffered saline (PBS) solution was purchased from Thermo Fisher Scientific (Rockford, Illinois, U.S.A.). Liquid nitrogen was used on the real *E. arborea* samples before extraction. Absolute ethanol was acquired from Carlo (Rouen, France) and the anti-fluorescein-peroxidase (anti-FITC-POD) was obtained from Roche Diagnostics (Basel, Switzerland).

Before use, the 20× SSPE buffer was diluted to a concentration of 2× using Milli-Q ultrapure water obtained from a Millipore purification system. All the reagents used in this assay were of analytical grade, so no purification was required.

For the DNA amplification by PCR, Taq Master Mix (2×), an optimized and ready-to-use PCR mixture of Taq DNA Polymerase, magnesium chloride ($MgCl_2$) 2 mM and

deoxynucleotide triphosphates (dNTPs) and PCR water were used. PCR Mastermix and water were acquired from Bioron (Römerberg, Germany).

2.2. Apparatus and Electrodes

The electrochemical genosensors were assembled using a SPGE (C223BT, DropSens) from ΩMetrohm (Oviedo, Asturias, Spain). The SPGE functioned as an electrochemical transducer. Moreover, an Autolab potentiostat (PGSTAT101, ΩMetrohm, Herisau, Switzerland) equipped with the NOVA 1.11.0 research software was used to measure all the electrochemical signals. The chronoamperograms were measured with a −0.1 V potential during 60 s and the current intensity utilized for the analytical analysis corresponds to the average of the last 10 s of the recorded current measurement. All the measurements were carried out at room temperature (25 °C ± 1.0 °C).

An iron mortar and pestle were utilized to extract the DNA from inside the heather flower plant and their DNA amplification of the targeted sequences was conducted using the conventional PCR technique. The design of the primers, specific nucleotide sequences that allow the amplification of the region of interest, was carried out using Primer-Blast (NCBI) [12] and purchased from Eurogentec (Seraing, Belgium). The protocol established by Bioron was employed for the preparation of the PCR mixtures, and the MyCycler™ thermal cycler from Bio-Rad Laboratories (Hercules, California, U.S.A.) was used for the amplification of the samples.

2.3. Oligonucleotides and Real DNA Samples

The synthetic probes designed for this study (Table 1) were purchased from Eurogentec as a lyophilized salt. All the oligonucleotide stock solutions were stored at −20 °C after being resuspended to 100 nM using Milli-Q ultrapure water. Working oligonucleotides were made daily by diluting the necessary concentration in 2× SSPE.

E. arborea and *Castanea sativa* leaves were obtained from the Botanical Garden of Porto (Portugal). Their genomic DNA was extracted by mechanically shredding the plants with liquid nitrogen in an iron mortar. The samples from *C. sativa* (the European chestnut tree) were submitted to the same process and used as a negative control.

Table 1. *Erica arborea* oligonucleotides.

Probes	5′→3′ Sequence	Base Pairs
Capture probe	GAC CTT CTT TTT AGG CCA ACC GAG CAC A	28
Signaling probe	GAC TGC GTA GCA TGC ACA ACG TGT CGC AGT TTG GCA ACC ACC ACT TGT TGT GAT GTC CGT CAT CAG G	70
Target probe	TGT GCT CGG TTG GCC TAA AAA GAA GGT CCC TGA TGA CGG ACA TCA CAA CAA GTG GTG GTT GCC AAA CTG TCG CGA CAC GTT GTG CAT GCT ACG CAG TC	98

2.4. Electrochemical Genosensor Design

The construction of the electrochemical genosensor involved four steps: pretreatment, the sensing phase, the sandwich hybridization reaction, and the electrochemical detection. Essentially, the pretreatment consists in cleaning the electrodes surface. Prior to use, all electrodes are washed with approximately 500 µL of ethanol and water, followed by drying under a nitrogen stream.

Then, during the sensing phase, a self-assembled monolayer (SAM) interface was established between the DNA-capture probe and the MCH spacer to guarantee the vertical orientation of the DNA sequences. In the first step, the DNA-capture probe is immobilized onto the SPGE and stored in a humified chamber overnight. The next day, the modified SPGEs are rinsed with the SSPE 2× buffer to remove weakly attached DNA-capture probes, followed by the addition of 3 µL of MCH to the SPGE.

The hybridization reaction unfolds in a two-stage process. Initially, a homogeneous hybridization occurs when the DNA-signaling probe connects to the DNA target. Subsequently, the partial hybridized DNA is added to the modified SPGE, enabling the complete hybridization between all three DNA sequences.

After 60 min, the SPGE are rinsed again with the buffer to eliminate any nonspecifically attached sequences. The sandwich hybridization format enhances assay selectivity by facilitating two distinct hybridization events: the homogenous hybridization between the target and the signaling probe and the subsequent binding of an anti-fluorescein antibody labeled with a horseradish enzyme to the fluorescein-labeled signaling probe.

To generate an ample electrochemical signal, POD enzymes are added onto the modified SPGEs, followed by a rinse after 30 min. Subsequently, the sensor is attached to the potentiostat, and 40 µL of TMB/H_2O_2 substrate is applied to the surface of the electrode for 1 min. The enzymatically oxidized product is then detected through chronoamperometry at –0.1 V for 60 s. The measurements are performed in triplicate for accuracy.

3. Results and Discussion

A sandwich format for the DNA-target probe was created using a semi-complementary fluorescein isothiocyanate-labelled DNA-signaling probe. To maximize the hybridization reaction, a mixed self-assembled monolayer of the heather-specific DNA-capture probe and mercaptohexanol was employed.

3.1. Optimization of the Analytical Parameters

The following analytical parameters: DNA, antibody and MCH concentrations and incubation times were optimized. Table 2 summarizes the results of the optimization processes of the analytical parameters.

Table 2. Selected analytical parameters levels used for the genosensor optimization.

Variables	Tested Range	Selected Value
DNA-capture concentration (µM)	0.25–10.00	1.00
MCH spacer concentration (µM)	0.00–1.00	0.50
MCH spacer incubation time (min)	5–30	5
Homogeneous hybridization incubation time (min)	15–60	30
Temperature (°C)	25–98	25
DNA-signaling concentration probe (µM)	0.13–0.50	0.50
Heterogeneous hybridization incubation time (min)	30–120	60
Antibody concentration (U/mL)	0.50–3.00	2.00
Antibody incubation time (min)	15–45	30

3.2. Analytical Characterization of the Optimized Genosensor

Using the selected values described in Table 2, the electrochemical genosensor was evaluated using the voltammetic technique: chronoamperometry. For this, increasing DNA-target concentrations, ranging from 0.03 to 5.00 nM were measured. A linear relationship (R^2 = 0.9981) between the blank-subtracted intensity current and the synthetic target concentration was obtained in the 0.03–2.00 nM range, with a slope and intercept value of 3.22 ± 0.03 (µA/nM) and 0.04 ± 0.01 (µA), respectively (Figure 1).

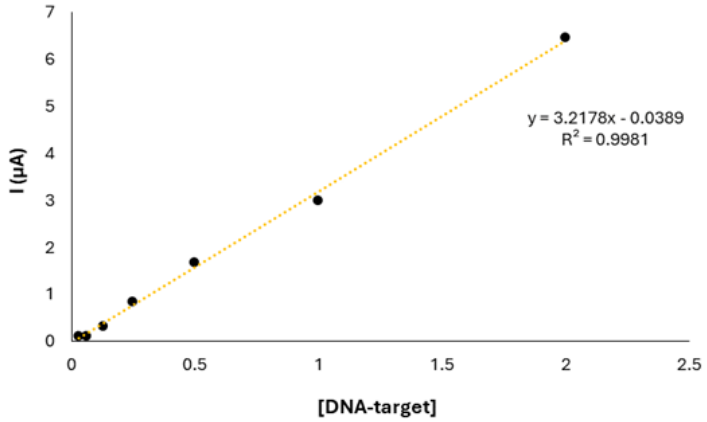

Figure 1. Calibration curve corresponding to the synthetic DNA-target concentrations ranging from 0.03 to 1.00 nM. Current responses obtained from an average of three replicates.

3.3. Evaluation of the Genosensor's Selectivity

Afterwards, the developed electrochemical genosensor was tested with PCR amplified samples of genomic DNA obtained from the *E. arborea* samples (Figure 2). The amplification of the targeted DNA was previously completed using the conventional PCR, according to the protocol established by Bioron (Römerberg, Germany).

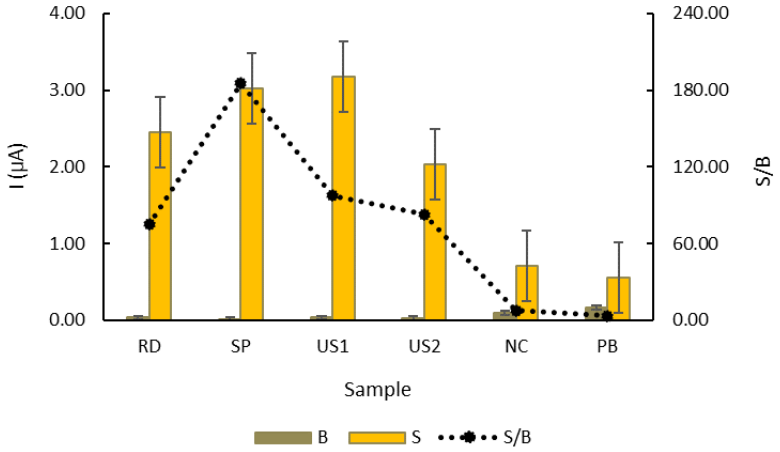

Figure 2. Correlation between the electrochemical signals detected from the complementary *Erica arborea* (RD, US1 and US2) and the non-complementary (NC and PB) amplified DNA to the synthetic DNA target (SP). Current intensity values of the blank assays (B) represented in brown, signal (S) in orange and the corresponding S/B ratio in black.

Under the previous conditions, the target discrimination, i.e., the sensor's selectivity was analyzed by comparing the chronoamperometric intensities obtained in the absence or presence of the synthetic DNA-target probe (with a concentration of 1 nM), 1 nM of the amplified *E. arborea* genomic DNA (RD), two samples of *E. arborea* of varying concentrations: 1.30 nM for sample US1 and 0.80 nM for sample US2 and with a noncomplementary DNA sample (NC) of another amplified plant species, *C. sativa*. Furthermore, to determine the influence of the primers on the developed electrochemical genosensor, a blank sample with the PCR products (PB) was also tested.

Also, analyzing the current intensity from the 1 nM genomic DNA sample (SP) to the 1 nM synthetic DNA probe there is a 6.10% difference between the two. This difference is acceptable and within the calibration curve variation. The responses registered by samples US1 and US2 are also within the calibration curve.

The highest current intensity was observed for the *E. arborea* DNA sequence with 1.30 nM (US1), followed by the synthetic DNA-target probe (SP). Nevertheless, the synthetic DNA presented the highest S/B value. On the other hand, the current responses from the NC (*C. sativa*) and PB sequences presented the lowest current intensities. These results indicate that this sensor design is a viable option to identify *E. arborea* DNA in real honey samples.

4. Conclusions

The high sensitivity and selectivity of the disposable electrochemical genosensor was achieved by designing a self-assembled monolayer (thiolated-DNA-capture probe and MCH spacer) and due to the design of the sandwich format assay, respectably. The amplification of the electrochemical signal conducted by the (POD) enzyme also influenced the genosensor's performance.

The developed sensor was successfully employed for the detection and quantification of E. arborea plant samples. This genosensor was able to detect with great selectivity both the synthetic and genomic DNA of the Erica arborea samples at different concentrations.

All optimizations contributed to enhance the sensor's sensitivity. Thus, electrochemical genosensors are a promising, innovative, easy-to-use and cost-effective tool to authenticate the origin of honeys, guaranteeing their quality and safety.

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