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Selective Electrochemical Determination of Caffeine at a Gold-Chitosan Nanocomposite Sensor: May Little Change on Nanocomposites Synthesis Affect Selectivity?

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Abstract

A simple and selective method for the determination of caffeine also in complex matrix has been developed at a gold electrode modified with gold nanoparticles (AuNPs) synthetized in a chitosan matrix in the presence of oxalic acid.

The electrochemical behaviour of caffeine at both gold bare and gold electrode modified with AuNPs with different morphology was carried out in acidic medium by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS). Electrochemical parameters were optimized in order to improve the electrochemical response to caffeine. The most satisfactory result, that means the higher electrochemical improvement, was obtained using a gold electrode modified with AuNPs synthetized in a chitosan matrix in the presence of oxalic acid, in aqueous solution containing $HClO_4$ 0.4 mol L^{-1} as supporting electrolyte.

The performance of the sensor was then evaluated in terms of linearity range ($2.0x10^{-6}$ - $5.0x10^{-2}$ mol L^{-1}, R = 0.999), operational and storage stability, reproducibility (RSD = 3.7%), limit of detection (LOD = $1.0x10^{-6}$ mol L^{-1}) and response to a series of interfering compounds as ascorbic acid, citric acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid, glucose, catechin and epicatechin.

The sensor was then successfully applied to determine the caffeine content in commercial beverages and results were compared with those obtained with HPLC-PDA as an independent method and with those declared from manufacturers.

Keywords

Gold Nanoparticles; Electrochemical Sensor; Caffeine; Interferences; Food Analysis

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1. Introduction

The versatility of gold nanoparticles has been well evidenced in the last decades, the application fields ranging from optics and electronics to biotechnology and catalysis [1-3]. This is due to their particular physical and chemical properties that may be tuned by varying the size and the shape of the core and modifying the surface with suitable functional molecules. In this context, increasing attention has been recently payed to chitosan as medium to synthetize and to stabilize gold nanoparticles (AuNPs) [4-7]. In fact, besides the biodegradable, biocompatible and non-toxic nature of this biopolymer, the presence of many functional amino and hydroxyl groups involves chitosan in the reduction process of the gold precursor to yield AuNPs that means not to use toxic reductants in the synthesis. In addition, the very good film-forming capability of chitosan allows high dispersion of AuNPs into the polymeric matrix. Such a film combines highly conductive AuNPs with organic functional groups, two properties making such a nanocomposite strongly suitable as material for electrochemical sensors.

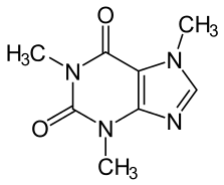
In a previous work, a novel green synthesis of gold-chitosan nanocomposites was developed, consisting briefly in reducing Au^{III} to Au^{0} in aqueous solution containing chitosan in the presence of different organic acids [8]. In the same work it was evidenced that AuNPs with different morphology were obtained varying the organic acid used in the synthesis process, suggesting the possibility that different electrochemical performance could arise from different AuNPs obtained with even if little changes in the synthesis. A first electrochemical sensor based on Au-chitosan nanocomposite film grown in the presence of acetic acid was already developed and successfully applied for selective electrochemical determination of caffeic acid in wines [9].

Those results encouraged further investigations on the possibility to develop other highly efficient electrochemical sensors for the determination of other specific analytes in complex matrices.

About that, caffeine (CAF, structure in the Scheme) is the most widely consumed psychoactive substance in human dietary, being present in many consumer products as coffee, tea, chocolate, soft and energy drinks. Many physiological effects of CAF are well known, from stimulation of the central nervous system, diuresis and gastric acid secretion [10] to nausea, seizures, trembling and nervousness [11]; mutation effects on DNA have been also reported [12]; finally, it is considered a risk molecule for cardiovascular diseases [13]. Recently, also an antioxidant activity has been suggested for CAF, showing protective effects against oxidative stress [14-16].

The presence of CAF in many beverages and drug formulations of worldwide economic importance [17] makes it an analyte of great interest and although many different analytical methods are currently applied, novel analytical methods for fast, sensitive and reliable determination of CAF are always necessary, especially for particular purposes, as the determination in specific matrix in the

presence of interfering agents, or in a specific concentration range, besides under beneficial conditions in terms of time-consuming, material cost and easy procedure [18].



Scheme. Caffeine structure

Nowadays, many separation methods are used for CAF determination as gas chromatography (GC) or liquid chromatography (LC) interfaced with mass spectrometry (MS) or photodiode array (PDA) detectors [19-25], with high sensitivity, selectivity and detection limit lower than 10^{-8} mol L^{-1}; spectroscopic methods in the UV-vis region [26] or infrared region (FTIR, NIRS) [27- 29] are also used because of the easy availability of colorimeters or spectrophotometers; even ^{1}H NMR spectrometry was used to determine CAF [30]. Most of these methods present different disadvantages, such as expensiveness, time-consuming and sample pre-treatment like extraction, pre-concentration and derivatization [31, 32] and finally skilled personnel is requested, often restricting their use in the routine analyses.

Electrochemical determination of CAF on different electrode materials, usually bare and miscellaneously modified carbon-based electrodes, has been also reported in literature [33-44]. The electrochemical methods offer a series of practical advantages as an easy procedure, not too expensive instruments, the possibility of miniaturization, besides good sensitivity, wide linear concentration range, predisposition for real-time detection and reduced sensitivity to matrix effects.

To the best of our knowledge, no study on the electrochemical behavior of CAF at a gold electrode or at a modified gold electrode has been published to date, even if literature reports the development of a biosensor based on a gold screen printed electrode modified with cysteamine [45] and another sensor based on molecularly imprinted polymers (MIPs) on a glassy carbon electrode modified with multiwalled carbon nanotubes (MWCNs) and gold nanoparticles (AuNPs) [46], both for the indirect determination of CAF.

In the present work, the electrochemical behavior of CAF has been studied in aqueous medium at a gold electrode both bare and modified with different Au-nanoparticles/chitosan nanocomposites (AuNPs), and then a sensor for fast and selective CAF determination has been developed. The range of linearity, the stability, the reproducibility, the response to interferents and the detection limit (LOD) of the sensor have been evaluated.

The sensor has been tested for the determination of CAF in a series of commercial beverages, in order to demonstrate its suitability for analyses of real samples. The content of CAF in the same commercial beverages has been also determined by HPLC-PDA as a more conventional and independent analytical method.

2. Experimental

2.1 Materials

Medium-molecular-weight chitosan (5800 g mol^{-1}), composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine with a degree of deacetylation of 75−85%, was purchased from Sigma-Aldrich. Acetic acid (AA), malonic acid (MA), oxalic acid (OA), tetrachloroauric acid ($HAuCl_4$), perchloric acid ($HClO_4$) 70%, sulphuric acid (H_2SO_4) 96%, formic acid, caffeine ReagentPlus®, chlorogenic acid (CGA), caffeic acid (CA), ferulic acid (FA), gallic acid (GA), cathechin (C), epicathechin (EC), glucose (GL), ascorbic acid (AsA), citric acid (CitA) were purchased from Sigma-Aldrich and used as received. All the chemicals were of analytical grade. HPLC grade acetonitrile was purchased from Carlo Erba (Milano, Italy); HPLC grade water was prepared with the Milli-Q purification system (Millipore, Vimodrone, Italy).

2.2 AuNPs/chitosan nanocomposites synthesis

The synthesis of chitosan-stabilized AuNPs was carried out in the presence of different carboxylic acid: acetic acid (AA), malonic acid (MA) and oxalic acid (OA), according to our recently published procedure [8, 9, 47]. The resulting samples were indicated as AuAA-CHIT, AuMA-CHIT and AuOA-CHIT, respectively, in general AuXX−CHIT, XX referring to the organic acid used in the synthesis, and characterized as previously reported [8, 9, 47].

2.3 Electrochemical measurements

Gold electrode (2 mm in diameter) was purchased from Metrohm Autolab (Utrecht, Netherlands). After the polishing steps, Au electrodes were modified by drop casting gold nanoparticles suspension as previously reported [8, 9, 47].

Electrochemical measurements were performed with an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie BV, Utrecht, Netherlands), using a conventional two-compartment three-electrode cell with a bare or modified Au working electrode, an Ag|AgCl electrode as reference and a Pt electrode as counter electrode. All the electrochemical experiments were carried out at room temperature on solutions of distilled-deionized water containing $5.0x10^{-3}$

mol L^{-1} CAF and 0.4 mol L^{-1} $HClO_4$ as supporting electrolyte without deaerating as dissolved oxygen does not interfere in the anodic potential window.

Cyclic voltammetry (CV) was carried out at the scan rate of 0.02 V s^{-1}.

Differential pulse voltammetry (DPV) parameters were optimized as follows: potential range from 0.30 V to 1.80 V, step potential 0.004 V, modulation amplitude 0.025 V, modulation time 0.05 s and scan rate of 0.02 V s^{-1}.

Electrochemical impedance spectroscopy (EIS) was performed with a frequency in the range 10^4 Hz - 0.1 Hz and a signal amplitude of 0.010 V, at the half peak potential (E_{ha}) of the analyte at both bare and modified electrode. Data were fitted by Z-view software (Scribner Associates, Inc.) using the Randles equivalent circuit [48] as a model for describing the electrolyte/electrode interphase, and consisting of Rs (bulk solution resistance) in series with a parallel combination of Rct (interfacial charge transfer resistance), Zw (diffusion of the analytes in solution), and Cdl (double layer capacitance). Rct data were obtained by fitting of all the experimental data through the Z-view program, using the method developed by Boukamp [49].

CV, DPV and EIS measurements were performed and elaborated by the Autolab NOVA 1.10 software system.

2.4 Caffeine calibration curve

CAF stock solutions were prepared daily at the final concentration of $5.0x10^{-2}$ mol L^{-1} and $5.0x10^{-3}$ mol L^{-1} in 0.4 mol L^{-1} $HClO_4$.

The calibration curve for CAF was constructed according to the standard addition method, using the average of six consecutive measurements for each addition of standard. The calibration curve was analyzed by linear least-square regression in OriginPro 8.1 (OriginLab Corporation, USA).

2.5 Real samples analysis

The content of CAF was estimated in commercial samples of Coca-Cola, Coca-Cola Light, Coca-Cola Zero, Pepsi, Red Bull and tea, properly diluted before measurement, the diluting process actually reducing the matrix effects of real beverages: samples of soft and energy drink were diluted 1:10 (v/v) with the supporting electrolyte, after sonical elimination of gas. Tea solutions were prepared by dissolving one teabag into 250 mL of boiling water and then diluted 1:10 (v/v) as the other commercial samples.

Standard addition method was employed for the analysis of all the beverage samples. The limit of detection (LOD) was obtained by using the equations LOD $=3s_{x/y}/b$ where $s_{x/y}$ and b were the estimated standard deviation and the slope of the analytical calibration function with a 95%

confidence level [50, 51]. The precision of all the electrochemical data at the different electrode was evaluated using seven electrodes (n=7).

2.6 HPLC-PDA instrumentation and conditions

Chromatographic separation was performed on an HPLC separation module 1525µ Waters (Milford, MA, USA) using a Waters XBridge C18 (150 x 2.1 mm i.d.) 5 µm analytical column, A (water/formic acid 0.02%) and B (acetonitrile/formic acid 0.02%) as mobile phase, and a flow rate of 0.20 mL min^{-1}. The 7 minutes isocratic elution was 10% B. After each analysis on real matrix, the column was washed with a binary gradient till 80% B and equilibrated to initial condition before the successive injection (5 µL injected). A Waters 996 photodiode array (PDA) detector was set for one spectrum per second in the range 220-600 nm.

The separation system was also linked to a Quattro Micro Tandem MS-MS with an electrospray ionization (ESI) source Waters (Micromass, Manchester UK) to monitor the real samples in full scan (data not shown). Data acquisition, data handling and instruments control were performed by MassLynx Software 4.1 v (Data Handling System for Windows, Micromass, UK).

A standard solution, prepared dissolving 1 mg mL^{-1} of CAF in acetonitrile, was diluted with the mobile phase A:B (90:10, v:v) to obtain 1, 5, 10, 15, 20 and 30 µg mL^{-1} working solutions.

The recorded PDA chromatograms were extracted at the CAF characteristic λ_{max} = 273 nm and the corresponding peak areas were measured. Calibration curve was forced through the origin and calculated with equal weighted least-squares linear regression analysis of peak area against standard nominal concentration (R = 0.996) by OriginPro 8.1 (OriginLab Corporation, USA).

Commercial samples of Coca-Cola, Coca-Cola Zero, Coca-Cola Light, Pepsi and Red Bull were degased, filtered (0.20 µm cellulose regenerated syringe filter, CPS Analitica, Milano), diluted with mobile phase A:B (90:10, v/v) and submitted to analytical determination. All the samples were diluted 1:10 except Red Bull, that was diluted 1:50. Solutions of commercial samples of tea, prepared as described above, were similarly filtered, diluted 1:10 with mobile phase and submitted to analytical determination. All the samples were analyzed in triplicate in three independent runs at room temperature, conditioned at 25°C.

3. Results and discussion

3.1 Electrochemical behavior of CAF at bare and modified gold electrode studied by CV, DPV and EIS

The electrochemical oxidation of CAF has been studied at different solid electrode [33-44], but no studies at a gold electrode have been published to date. Gold electrode material possesses very

attractive characteristics: high thermal conductivity, a large potential window, low adsorption capability, low and stable background current, electrochemical stability at different pH.

Cyclic voltammetries of $5.0x10^{-3}$ mol L^{-1} CAF were carried out at both bare and modified Au electrode in aqueous medium in the presence of different strong acids as supporting electrolytes, including H_2SO_4 and $HClO_4$, in the pH range 0.5–3.0. CV data at bare and modified electrode in 0.4 mol L^{-1} $HClO_4$ and in 0.1 mol L^{-1} H_2SO_4 were reported in Tables 1 and A1 (Supplementary material), respectively. In agreement with literature [37], the current signal increased with decreasing pH, evidencing that acidic media (pH < 3.0) are the most suitable for analytical purposes. In particular, the highest anodic current was found in the presence of $HClO_4$ at pH = 0.4. $HClO_4$ 0.4 mol L^{-1} was then chosen as the optimum supporting electrolyte and used in further experiments.

The effect of modifying the electrode surface on the oxidation process of CAF was then evaluated. As shown in Figure 1 and Table 1, the anodic peak potential (E_{ap}) value appeared not significantly affected by modifying the electrode, while it was evident the electroanalytical improvement concerning the higher anodic peak current intensity at the AuNPs modified electrode.

Table 1. Cyclic Voltammetry (CV) data of caffeine in 0.4 mol L^{-1} $HClO_4$ at bare and AuNPs modified gold electrode. [CAF] = $5.0\ x10^{-3}$ mol L^{-1}, scan rate 0.02 V s^{-1}; reference electrode Ag/AgCl.

Au electrode	E_{ap} (V)	ΔE_{ap} (V)[a]	I_{ap} (µA)	ΔI_{ap} %[b]
Bare	1.55	-	36.49	-
AuAA-CHIT	1.55	0.00	47.44	30
AuOA-CHIT	1.52	-0.03	72.91	100
AuMA-CHIT	1.53	-0.02	46.71	28

[a] ΔE_{ap} was the difference between E_{ap} at bare electrode and E_{ap} at modified one.

[b] ΔI_{ap} was calculated using the equation : $(I_{apM} - I_{apB}/I_{apB}) \times 100$, where I_{apM} and I_{apB} are the anodic current intensity at modified and bare Au electrode, respectively.

The advantages of sensing interfaces modified with AuNPs, compared to Au unmodified and to non-Au sensing interfaces, are well-known [8, 47]: they consist of an increased sensing surface, an improved electrical connectivity through an AuNPs 3D network and a highest chemical accessibility to the analyte through the same network [52].

Different AuNPs, indicated above as AuXX−CHIT, have been tested: the most pronounced amplification of the electrochemical response was observed for AuOA-CHIT (ΔI_{ap} = 100 %), whereas a current intensity increase of only 30 % and 28 % was observed for AuAA-CHIT and AuMA-CHIT, respectively.

The high performance of AuOA-CHIT modified electrode can probably be assessed to a better interconnection between AuNPs and caffeine: the 3D network of AuNps in the chitosan film can be considered responsible for a more efficient electron transfer, whereas the functional groups on the chitosan can interact chemically with CAF as scavengers.

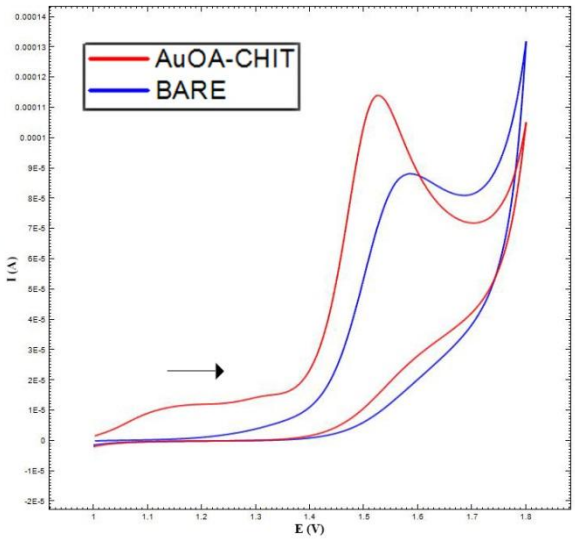


Figure 1. Cyclic voltammograms (CVs) of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ recorded at bare electrode (blue) and at AuOA-CHIT modified gold electrode (red); scan rate 0.02 V s^{-1}; reference electrode Ag/AgCl.

It is interesting to note that the same AuOA-CHIT nanocomposite had produced a different response with a different analyte as caffeic acid. In fact, the higher amplification of the current intensity and the consequent best performance towards caffeic acid had been obtained with AuAA-CHIT modified electrode, as previously reported [8], while only an increase of 20% had been observed with AuOA-CHIT modified electrode. So, different AuXX-CHIT can act as a selective scavenger toward the specific target molecule.

AuOA-CHIT modified electrode has been chosen for further experiments targeted to the development of a selective sensor for caffeine determination.

CV of $5.0x10^{-3}$ mol L^{-1} CAF in aqueous solution 0.4 mol L^{-1} $HClO_4$ evidenced an irreversible anodic peak at both bare and AuOA-CHIT modified electrode at E_{ap} +1.55 V and +1.53 V vs.

Ag/AgCl, respectively. The E_{ap} value is in agreement with data previously reported in literature [33-44, 53] as well as the irreversibility of the anodic process of CAF in aqueous medium. In fact, in aqueous solution the radical cation obtained from the first monoelectronic oxidation of CAF can easily react with water and the product can undergo further oxidation to give the substituted uric acid with a $2e^-$, $2H^+$ whole process [36-38]. This last product can be furtherly oxidized, the overall process resulting in a $4e^-$, $4H^+$ oxidative path. These chemical reactions following the primary oxidation cause the irreversibility of the anodic process of CAF in aqueous medium. The pH value influence on the E_{ap} value was also in agreement with the proton-involving mechanism: the peak potential shift towards more positive values at pH < 3.0 suggested a more difficult oxidation, likely due to the protonation of the N-atom involved in the primary anodic process.

The influence of the scan rate on the anodic peak current was also evaluated. CVs of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ were recorded at different scan rates from 0.01 to 0.20 V s^{-1}. The anodic peak currents resulted linearly proportional to the square root of the scan rate both at bare and modified electrode, following the linear regression equation $I_{ap} = -0.015 + 0.71\ \nu^{1/2}$ (I_{ap} in μA, ν in V s^{-1}, R = 0.995) for AuNPs modified gold electrode, and $I_{ap} = 0.010 + 0.55\ \nu^{1/2}$ (I_{ap} in μA, ν in V s^{-1}, R = 0.995) for bare electrode, indicating that the electrochemical oxidation of CAF at Au-Electrode is a diffusion-controlled process. The logarithm analysis of the anodic peak current I_{ap} vs the scan rate ν also supports a diffusion-controlled process, as evidenced by the equations $\lg I_{ap} = -2.77 + 0.50 \lg \nu$ (R = 0.998) and $\lg I_{ap} = 1.31 + 0.48 \lg \nu$ (R = 0.999) at bare and modified electrode, respectively (I_{ap} in μA, ν in V s^{-1}).

DPV of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ was performed as a more sensitive voltammetric technique to investigate the dependence of peak current from concentration. Modulation amplitude, modulation time and scan rate were optimized. An increasing peak current was found for increasing modulation amplitude, the same occurring for the peak width. As the peak became too much wider for modulation amplitude higher than 0.025 V, the optimized value of 0.025 V was chosen for modulation amplitude. A decreasing peak current was found for increasing modulation time: the most stable peak current was observed at 0.05 s.

CAF oxidation was further investigated by EIS in the presence of 0.4 mol L^{-1} $HClO_4$ and 0.1 mol L^{-1} H_2SO_4. EIS data were in good agreement with the voltammetric behavior (see Table 2 and Figure 1).

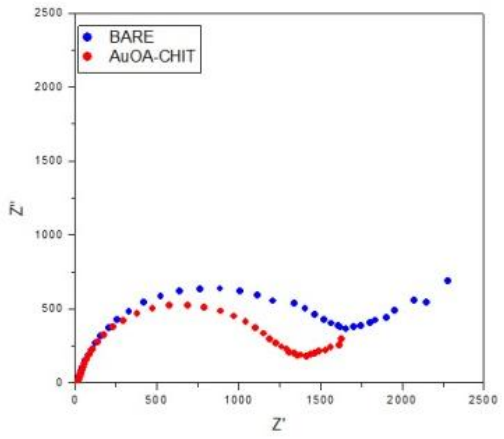


Figure 2. Electrochemical impedance spectra (EIS) of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ recorded at bare electrode (blue) and at AuOA-CHIT modified gold electrode (red), frequency range 10^4 Hz-0.1Hz, signal amplitude of 0.010 V, open circuit voltage (OCV) conditions.

In particular, the smallest charge transfer resistance (Rct) was observed at AuOA-CHIT modified Au electrode in 0.4 mol L^{-1} $HClO_4$, with the value 1.43 KΩ vs 1.90 KΩ for bare electrode, while Rct in 0.1 mol L^{-1} H_2SO_4 remained unchanged, 1.90 KΩ for bare electrode vs 1.93 KΩ for AuOA-CHIT modified one: the smallest Rct of modified Au electrode in $HClO_4$ indicates less opposition to the passage of electric current, as expected from the voltammetric data evidencing an increasing of peak current at the AuOA-CHIT modified electrode. The Relative Standard Deviation (RSD %) of all electrochemical data of CV and EIS recorded on CAF $5.0x10^{-3}$ M, using seven electrodes, were reported in Tables A2, A3 and A4 (Supplementary material).

Table 2. EIS data of CAF at bare and AuOA-CHIT modified gold electrode in 0.4 mol L^{-1} $HClO_4$ and 0.1 mol L^{-1} H_2SO_4 as different electrolyte. [CAF] = 5.0 $x10^{-3}$ mol L^{-1}; reference electrode Ag/AgCl.

Electrolyte	**Bare Au electrode**		**AuOA-CHIT**	
	E_{ha} (V)	**Rct (KΩ)**	**E_{ha} (V)**	**Rct (KΩ)**
0.4 mol L^{-1} $HClO_4$	1.27	1.90	1.32	1.43
0.1 mol L^{-1} H_2SO_4	1.23	1.90	1.29	1.93

[a] E_{ha} was the half peak potential of the analyte at bare and at modified electrode.

The regenerability of the modified electrode was also tested. It is well known that CAF oxidation at carbon electrode gives rise to adsorption [18]. The fouling phenomena on the nanocomposite-modified electrode were examined and it was found that CAF weakly adsorbed on the nanocomposite and easily desorbed from the nano-surface. In fact, when the same nanocomposite electrode previously used for CAF determination was "cleaned" by anodic scan in the electrolyte solution not containing CAF, a weak voltammetric signal corresponding to the oxidation of CAF was first evidenced but it quickly disappeared in the successive scans. Hence, the electrode surface can be easily cleaned and regenerated after measuring by scanning twice or three times in the range +0.90 / +1.80 V in fresh electrolyte solution.

3.2 Calibration curve and analytical parameters

An average of six consecutive DPV measurements with the optimized parameters was used for the calibration curve. DPV voltammograms at various CAF concentrations in 0.4 mol L^{-1} $HClO_4$ are shown in Figure 3. A good linearity of the peak current values vs. CAF concentrations was found in the range $2.0x10^{-6}$ - $5.0x10^{-2}$ mol L^{-1}, according to the equation $I_{ap} = 7.3 + 8.0\ C_{CAF}$ ($R = 0.992$, I_{ap} in μA, C_{CAF} as mmol L^{-1}). The limit of detection (LOD) for CAF, calculated as described above, was found to be $1.0x10^{-6}$ mol L^{-1}.

The repeatability of the DPV measurements was tested by constructing 10 successive calibration plots for CAF with the same sensor, in the linearity range $2.0x10^{-6}$ - $5.0x10^{-2}$ mol L^{-1}. A relative standard deviation (RSD) value of 2.5% indicated a good repeatability with no need to apply a cleaning or regeneration procedure for the sensor.

The fabrication reproducibility of the sensor was evaluated for seven electrodes (n=7) on 2.0 x 10^{-5} mol L^{-1} CAF solution. A RSD of 3.7% evidenced a reliable construction procedure of the sensor.

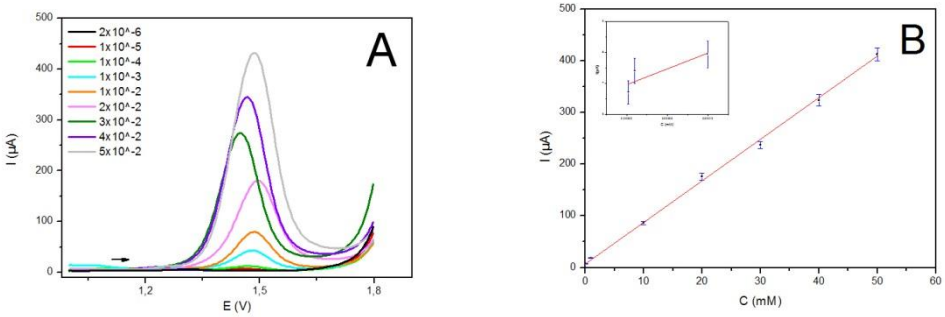


Figure 3. A) Differential pulse voltammograms (DPVs) of CAF in 0.4 mol L^{-1} $HClO_4$ at AuOA-CHIT modified gold electrode, step potential 0.004 V, modulation amplitude 0.025V, scan rate 0.02 V s^{-1}; reference electrode Ag/AgCl. CAF concentration range is $2.0x10^{-6}$ - $5.0\ x10^{-2}$ mol L^{-1}; B) the calibration curve with the inset showing the corresponding lower concentrations.

The operational stability was evaluated by repeating once an hour the measurements with the sensors (n=5) working continuously in a solution of caffeine $5.0x10^{-4}$ mol L^{-1} for 10 h. A continuous average decrease of the response with time was observed, till a value of 25% after 10 hours.

The storage stability of the sensors was evaluated at +4° C under wet conditions storage as well as at room temperature (RT) under dry conditions. The response to $5.0x10^{-4}$ mol L^{-1} CAF of five sensors stored at +4°C under wet conditions was tested every 3 days for 80 days: the average response decreased of 15% with respect to the initial signal after the first two weeks, remaining quite constant for 30 days, and then continuously decreasing till a final value of 35% of the initial signal after 80 days from the beginning. The response to $5.0x10^{-4}$ mol L^{-1} CAF of five sensors, stored at RT in dry conditions, was tested every 3 days for 1 month. After this period, the average response showed a decrease of 50%.

3.3 Interferences

The effect of possible interfering compounds was evaluated, at both bare and modified electrode. The species tested were ascorbic acid (AsA), caffeic acid (CA), citric acid (CitA), ferulic acid (FA), gallic acid (GA), chlorogenic acid (CGA), glucose (GL), catechin (C) and epicatechin (EC), usually found in beverages containing CAF. A concentration ratio between interfering compounds and CAF of 100:1 was tested.

DPV curves were recorded in 0.4 mol L^{-1} $HClO_4$ containing $5.0x10^{-4}$ mol L^{-1} CAF in the absence and in the presence of the interfering compound. Results are shown in Figure 4.

AsA interfered with the CAF response, increasing the oxidation peak at bare electrode of 4% and decreasing it of 1% at the modified one.

CitA did not appear to interfere with the determination of CAF, at both bare and modified electrode.

GL was tested as possible interferent, the consumption of CAF being often associated with the addition of common sugars. A decrease of the response at both the electrodes was observed, probably because of the formation of a sugar-caffeine complex [54]. C, EC and GA were considered as interfering agents because present in tea. GA showed a slight decrease of the peak current at both the electrode, while C and EC did not show a significant signal at modified electrode, as also previously reported [47], so they could not interfere with CAF detection. A slight decrease of the peak current was observed at the bare electrode for C and EC.

CA, CGA and FA are antioxidants present in coffee and tea. It has long been known that CAF interacts with polyphenolic molecules in aqueous solution [55] and for this reason we have

considered the influence of hydroxycinnamic acids on CAF detection. As shown in Figure 4, no significant interaction was observed, the signal only slightly decreasing at both the electrodes, bare and modified.

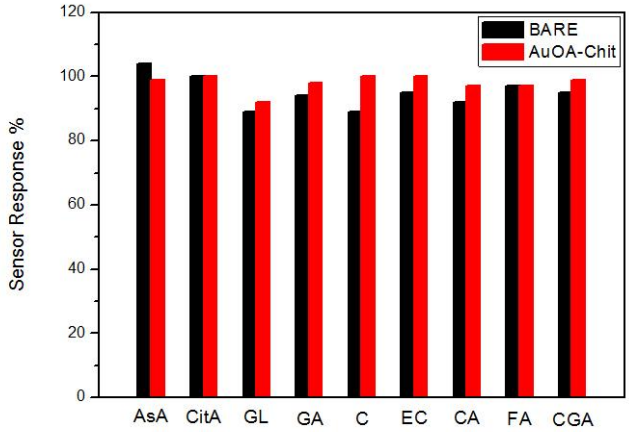


Figure 4. Histogram representing the effect of the following interferences at both bare (black) and at AuOA-CHIT modified electrode (red): ascorbic acid (AsA), citric acid (CitA), glucose (GL), gallic acid (GA), catechin (C), epicatechin (EC), caffeic acid (CA), ferulic acid (FA) and chlorogenic acid (CGA).

3.4 Measurements in commercial samples

CAF was determined in commercial samples by standard addition method. The commercial samples were only properly diluted with the supporting electrolyte solution. The 10-fold dilution for tea and for soft and energy drinks was found the optimum.

The content of CAF in the same commercial samples was also determined by HPLC-PDA as an independent method. By comparison with the analytical standard, the peak of CAF was identified by retention time t_R = 4.11± 0.10 min, showing a very good and a negligible matrix effect. The peak of CAF was furtherly identified by monitoring the molecular mass in positive ionization mode $[M+H]^+$ at *m/z* 195.2 (data not shown).

The purity peak calculated by MassLynx Software was 99/100 % for all samples, evidencing no interference by the other components of the analyzed samples. CAF amounts determined in the analyzed commercial beverages were presented as mean ± SD, as reported in Table 3.

Table 3. Determination of CAF in real samples (n=3) of soft and energy drinks and teas, diluted with 0.4 mol L^{-1} $HClO_4$, at AuOA-CHIT modified electrode and with an HPLC- PDA method.

Beverage sample	Proposed sensor	SD	Method HPLC-	SD	Declared by

	(mg/L)	(%)	PDA (mg/L)	(%)	manufacturer (mg/L)
Coca Cola	95.9	2.0	117.6	2.0	96.1
Coca Cola Light	120.7	3.1	113.8	4.7	129.9
Coca Cola Zero	98.5	4.2	109.4	4.7	96.0
Pepsi Cola	107.1	3.4	104.5	3.0	107.3
Red Bull (energy drink)	318.4	8.7	321.0	28.2	320.0
Lipton Green Tea	147.2	7.1	245.0	20.6	148.3
Lipton Yellow Label Tea	233.1	6.5	279.8	25.4	233.0

Satisfactory results were obtained with the AuOA-CHIT modified electrode for the determination of CAF in tea, soft and energy drink samples, the found values being in a very good agreement with the content declared by manufacturers. A generally good agreement with HPLC-PDA data was also found, the stronger difference being on tea samples. It is noteworthy that the most important divergence occurs on a natural matrix as tea is, more complex for its own nature and rich in polyphenols, so that interfering compounds could play a crucial role in both methods, electrochemical or chromatographic as well.

3.5 Comparison with other caffeine sensors

Several analytical methods have been reported in the literature employed for the detection of CAF from LC to electrochemical ones. The most common analytical parameters, such as linearity range, LOD and the application media, of a series of literature data are compared and resumed in Table 4. Most of the analytical methods for CAF determination showed a very limited linearity range, generally two or three orders of magnitude, confining the field of application to specific matrices, e.g., only soft or energy drinks, or teas or coffee or cola beverages, and finally drugs, human fluid and waste waters. LOD values appeared in many cases in the range 10^{-8} - 10^{-6} mol L^{-1} [20, 21, 23, 25, 26, 31-35, 37-39, 56]. In other papers, lower LOD in the range 10^{-11} - 10^{-9} mol L^{-1} were reported [19, 22, 24, 41, 42] but with restricted linearity ranges making the sensor not applicable to different matrices. It is to remark that in three cases [27, 40, 53] a LOD concentration was not mentioned.

On the other hand, in many cases no comparison with an independent method was reported [see for example 37, 43, 44, 53] while in others the study of interferences was not comprehensive,

considering that CAF should need to be determined in completely different media, from waste waters [19, 22] to human fluid, drugs or beverages [see for example 21, 34, 35, 36, 39, 40, 42-44].
In the present work, a sensor with a four orders of magnitude linearity range has been developed, a LOD suitable for CAF detection in synthetic as well as natural beverages has been reported, and an accurate study of interfering compounds has been carried out for CAF determination also in complex matrices. Finally, the obtained results of CAF contents have been compared, showing a good agreement, with those obtained with an independent method as HPLC-PDA and with the CAF contents declared by the manufacturers.

Table 4. An overview of analytical methods for CAF determination

Detection method	Linearity range (mol L^{-1})	LOD (mol L^{-1})	Application	Reference
Gas Chromatography-Mass Spectrometry (GC-MS)	$2.60x10^{-8}$ - $1.54x10^{-4}$	$5.15x10^{-9}$	Soft drinks	19
	$5.15x10^{-8}$ - $1.03x10^{-5}$	$3.44x10^{-8}$	Wastewaters	20
	$5.14x10^{-6}$ - $4.12x10^{-4}$	$5.14x10^{-7}$	Human serum	21
Liquid Chromatography (LC)	Not declared	$2.05x10^{-11}$	Wastewaters	22
High Performance Liquid Chromatography (HPLC)	$8.20x10^{-7}$ - 0.013	$2.57x10^{-7}$	Caffeine drinks	23
	$5.15x10^{-6}$ - 0.0026	$1.44 x10^{-10}$	Teas and dietary supplements	24
	$2.57x10^{-8}$ - $1.01x10^{-6}$	$2.16 x10^{-8}$	Teas, instant coffee, mate, soft and energetic drinks	25
	0.0061 - 0.56	$1.8x10^{-6}$	Tea	32
Electrospray ionization - ion mobility spectrometry (ESI-IMS)	$2.57x10^{-6}$ - $1.03x10^{-4}$	$1.02x10^{-6}$	Green tea and human plasma	31
FT-NIR spectroscopy	0.00 - $1 x10^{-3}$	Not declared	Teas and coffees	27
Near Infrared Spectroscopy (NIRS)	0.95 - 4.13 g/100g	$4.6x10^{-5}$	Coffees	29
UV-Vis spectroscopy	$1.25x10^{-5}$ - $1.42x10^{-4}$	$8.50 x10^{-6}$	Coffee beans	26
FT-IR spectroscopy	Not declared	$4.63x10^{-5}$	Coffees	20
1H NMR	$3.09x10^{-4}$ - 0.005	$2.73x10^{-4}$	Soluble coffees	30

Electrochemical Methods				
Cyclic Voltammetry (CV) WE bare boron doped diamond electrode (BDDE)	$1.00x\ 10^{-6}$ - $4.00x10^{-4}$	Not declared	Instant and tinned coffees, cola beverage	53
Linear Sweep Voltammetry (LSV) WE poly(safranine T)/glassy carbon electrode (GCE)	$3.00x10^{-7}$ - $1.00x10^{-4}$	$1.00x10^{-7}$	Teas	35
Differential Pulse Voltammetry (DPV) WE Nafion/GCE	$9.95x10^{-7}$ - $1.06x10^{-5}$	$7.98x10^{-7}$	Cola beverages	33-34
Differential Pulse Voltammetry (DPV) WE Nafion/GCE	$4.00x10^{-8}$ - $7.00x10^{-3}$	$4.00x10^{-8}$	Soft and energy drinks, instant coffee	44
Differential Pulse Voltammetry (DPV) WE Nafion/Graphene Oxide (GO)/GCE	$4.00x10^{-7}$ - $8.00x10^{-5}$	$2.00x10^{-7}$	Soft and energy drinks, cola beverage	56
Differential Pulse Voltammetry (DPV) WE bare boron doped diamond electrode (BDDE)	$4.00x10^{-7}$ - $2.50x10^{-5}$	$1.50\ x10^{-7}$	Soft and energy drinks, teas, coffees	37
Differential Pulse Voltammetry (DPV) WE Molecularly Imprinted Polymer (MIP)/Carbon Paste Electrode (CPE)	6.00×10^{-8} - 2.50×10^{-5}	$1.50x10^{-8}$	Soft drinks	38
Differential Pulse Voltammetry (DPV) WE Molecularly Imprinted Siloxane (MIS)/Multi Walled Carbon Nanotubes(MWCNTs)/VinylTrimethoxySilane (VTMS) /GCE	7.50×10^{-7} - 4.00×10^{-5}	$2.20\ x10^{-7}$	Coffees, energy drinks,	39
Differential Pulse Voltammetry (DPV) WE Electrochemically reduced graphene(ERGO)/GCE	$5.00x10^{-5}$ - $3.00x10^{-4}$	Not declared	Cola beverage, tea and soluble coffee	40

Differential Pulse Voltammetry (DPV) WE anisotropic gold nanoparticle–chitosan–ionic liquid/graphene/GCE	$2.50x10^{-8}$ - $2.49x10^{-6}$	$4.42x10^{-9}$	Energy drink, teas, drugs	41
Differential Pulse Voltammetry (DPV) WE electrochemically pre-treated graphite pencil electrode (PGE)	$1.00x10^{-6}$ - $9.00x10^{-6}$	$9.81x10^{-9}$	Human plasma and urine	42
Differential Pulse Voltammetry (DPV) WE polypyrrole sol-gel and gold nanoparticles hybrid nanocomposite modified pencil graphite electrode (PGE)	$2.00x10^{-9}$ - $5.00x10^{-8}$ $5.00x10^{-8}$ - $1.00x10^{-6}$	$9.00x10^{-10}$	Soft and energy drinks, green tea, human plasma, drugs and urine	42
Differential Pulse Voltammetry (DPV) WE gold nanoparticle–chitosan / Gold electrode (AuE)	$2.00x10^{-6}$ - $5.00x10^{-2}$	$1.00x10^{-6}$	Cola beverages, energy drink, teas	This work

4. Conclusions

In contrast to classical approaches, electrochemical sensors show very interesting performances and in particular the electrode material plays a challenging and central role. Gold electrode material possesses various desirable properties, such as high thermal conductivity, electrochemical stability in both alkaline and acidic media, very low and stable background current as well as a wide usable potential range, and low adsorption ability.

The lecture key of the present work should be searched into the possibility to obtain performant sensors selective for specific target molecules using gold electrode modified with AuNPs with different morphology obtained through little changes in a simple synthetic pathway.

A gold electrode modified with an Au-chitosan nanocomposite film grown in the presence of acetic acid had shown a good response to caffeic acid and a sensor had been developed and applied for selective determination in wines, as previously reported [9]. The same electrode did not result performant to a different analyte as caffeine, while a surprising response has been evidenced with a gold electrode modified with Au-chitosan nanocomposite grown in the presence of oxalic acid.

So a performant sensor based on Au-chitosan nanocomposite film grown in the presence of oxalic acid has been developed for selective determination of caffeine and successfully applied also in complex matrix.

Besides the very good response towards an analyte of high interest as caffeine is, the developed sensor can be easily regenerated after the use, the electrode material is cheaper and higher available with respect to other ones like BDD, its selectivity allows to neglect the interference of molecules widely distributed, especially in natural beverages, such as ascorbic acid, glucose, catechin, epicatechin, gallic acid, caffeic acid, chlorogenic acid and ferulic acid.

Furthermore, the proposed method based on differential pulse voltammetry is simple, fast and affordable and a minimum manipulation of the sample is necessary as dilution with acidic medium.

Last, the wide dynamic range allows determination of caffeine in beverages differently containing it.

Further studies are necessary to improve the sensing platform sensitivity in order to detect caffeine in other complex matrices such as decaffeinated coffee and tea or drugs.

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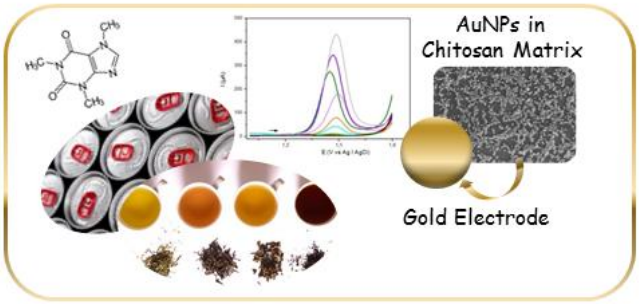
Captions of Figures

Figure 1. Cyclic voltammograms (CVs) of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ recorded at bare electrode (blue) and at AuOA-CHIT modified gold electrode (red); scan rate 0.02 V s^{-1}; reference electrode Ag/AgCl.

Figure 2. Electrochemical impedance spectra (EIS) of $5.0x10^{-3}$ mol L^{-1} CAF in 0.4 mol L^{-1} $HClO_4$ recorded at bare electrode (blue) and at AuOA-CHIT modified gold electrode (red), frequency range 10^4 Hz-0.1Hz, signal amplitude of 0.010 V, open circuit voltage (OCV) conditions.

Figure 3. A) Differential pulse voltammograms (DPVs) of CAF in 0.4 mol L^{-1} $HClO_4$ at AuOA-CHIT modified gold electrode, step potential 0.004 V, modulation amplitude 0.025V, scan rate 0.02 V s^{-1}; reference electrode Ag/AgCl. CAF concentration range is $2.0x10^{-6}$ - $5.0x10^{-2}$ mol L^{-1}; B) the calibration curve with the inset showing the corresponding lower concentrations.

Figure 4. Histogram representing the effect of the following interferences at both bare (black) and at AuOA-CHIT modified electrode (red): ascorbic acid (AsA), citric acid (CitA), glucose (GL), gallic acid (GA), catechin (C), epicatechin (EC), caffeic acid (CA), ferulic acid (FA) and chlorogenic acid (CGA).



Graphical abstract

Highlights

- An electrochemical method fast, simple and affordable is proposed.
- A performant sensor in terms of selectivity, stability, response time was developed.
- The nanomaterial plays a challenging role thanks to its regenerability and selectivity.
- A minimum manipulation of real samples as tea, soft and energy drinks was requested.