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PORTABLE ELECTROCHEMICAL SENSOR BASED ON 4-AMINOBENZOIC ACID-FUNCTIONALIZED HERRINGBONE CARBON NANOTUBES FOR THE DETERMINATION OF ASCORBIC AND URIC ACID IN HUMAN FLUIDS

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ABSTRACT

A new portable electrochemical sensor based on 4-aminobenzoic acid-modified herringbone carbon nanotubes (hCNTs-4ABA/Au-IDA) has been developed for the simultaneous determination of ascorbic acid (AA) and uric acid (UA) in physiological fluids. AA and UA were quantified by chronoamperometry at 0.1 and 0.32 V, respectively, in phosphate buffer solution (PBS 0.25 M, pH 7.0). Significant results were obtained for the separate quantification of AA and UA, with a limit of detection (LOD) of 0.65 μM for both analytes, and sensitivities of $(9.0 \pm 0.4) \text{ A g}^{-1} \text{ mM}^{-1}$ and $(8.8 \pm 0.3) \text{ A g}^{-1} \text{ mM}^{-1}$ for AA and UA, respectively. Repeatability was studied at 50 μM for AA and UA, providing relative standard deviations (RSD) lower than 9%. Additions of glucose, dopamine and epinephrine did not interfere with the AA and UA determination. Furthermore, UA did not interfere with AA determination at 0.1 V, although AA additions increased the current recorded at 0.32 V. The method has been successfully applied to human urine, perspiration and serum samples, without significant matrix effects, which allows for the use of an external calibration and analysis of all the matrices investigated.

Keywords: functionalized carbon nanotubes, ascorbic acid, uric acid, portable electrochemical sensor, human fluids

1. Introduction

Ascorbic acid (AA) and uric acid (UA) are of great importance to the physiology of living organisms given that they are involved in several biological functions. Recent decades have witnessed an increase in studies aiming to determine accurate AA and UA determination methods due to the fact that concentration levels of these analytes in physiological fluids may be makers of disease [1,2]. Additionally, the interest in determining AA levels in human fluids lies in our inability to synthesize it [3]. Therefore, the accurate quantification of these analytes is crucial for disease diagnosis [4,5]. In this context electrochemical methods, which are also low-cost, fast and provide high sensitivities, provide a suitable option [6,7].

Carbon materials are one of the most widely used materials in electrochemical applications because of their outstanding properties [8,9]. Particularly, carbon nanotubes (CNTs) are excellent electrode materials due to their high electrical conductivity, high surface area, high mechanical resistance and modifiable surface chemistry, as well as their relative chemical inertness in most electrolytes [10–13]. It is noteworthy that herringbone carbon nanotubes (hCNTs) have a large number of edge-plane-like defect sites, which are responsible for much of their chemical and electrochemical reactivity [12,13]. Thus, hCNTs are considered a good choice for electrochemical sensors development as analytes can become attached to a larger number of sites. The main drawback of CNTs is that they aggregate in almost all aqueous and organic solvents [10,14]. Consequently, research into CNT functionalization has increased in the last decade in an endeavor to improve their dispersibility, thereby extending their applications [12,15–20].

Another purpose of CNT functionalization is to tailor their properties, which offer several advantages for catalysis, analysis and biological applications [12,21]. There are several strategies for modifying the CNTs surface, among which electrochemical functionalization provides an efficient, clean and more versatile alternative [18,22]. Even though the role of nitrogen functionalities is not well understood yet, it has been demonstrated that CNT

functionalization with nitrogen groups [23,24] could enhance their sensitivity for AA and UA quantification. This may be ascribed to a delocalized conjugated system formed with the sp^2 -hybridised carbon frameworks, attributed to the lone electron pairs of nitrogen atoms. At the same time, nitrogen functionalization provides a large number of sites for the AA or UA attachment by hydrogen bonds, which may activate the amine groups and accelerate the charge transfer between the analytes and the carbon material [25,26]. A promising candidate for the hCNTs functionalization is the 4-aminobenzoic acid (4-ABA), which has been employed for CNT functionalization, showing suitable results for nitrogen contents and electroactivity in oxygen reduction reaction [20].

Functionalized and non-functionalized CNTs modified electrodes have been employed for the electroanalytical determination of several compounds. For instance, simultaneous or individual determination of AA and UA has been successfully achieved with multiwalled carbon nanotubes (MWCNTs) [27], pristine CNTs [28], functionalized CNTs (either with polymers [29–32] or metal nanoparticles [33,34]). Furthermore, the synergistic effect between several carbon materials has been evaluated by mixing graphene oxide (GO) and CNTs [35], or graphene and CNTs [36], among others. However, one of the drawbacks of these sensors is that they have been developed by modifying a glassy carbon (GC) or using a carbon paste-based electrode, which impedes the development of portable electrochemical devices. There are few studies into screen-printed CNT-modified electrodes for the individual determination of AA [37–39], UA [40], and for the simultaneous quantification of both AA and UA [41]. Moreover, graphene has been used to manufacture interdigitated electrodes for this application [42]. Recently, our research group has employed riboflavin-stabilized graphene to quantify UA with and without AA [43]. Also, gold nanoparticles (Au-NPs) supported on GO have been synthesized to modify an interdigitated microelectrode array, allowing the simultaneous determination of AA and UA by chronoamperometry [44].

Another disadvantage of the aforementioned sensors is that they have been calibrated by the standard addition method due to matrix effects. In order to overcome these drawbacks, we present a portable sensor based on 4-ABA-modified hCNTs for the simultaneous quantification of AA and UA in human biological samples (i.e. urine, perspiration and serum) without matrix effects. This portable sensor is an improvement on the devices developed in previous studies, where external calibration could be applied, thereby saving resources (i.e. time, sample and reagents) in routine analysis.

2. Experimental

2.1. Reagents and equipment

Herringbone carbon nanotubes (hCNTs) were purchased from TCI Chemicals. Uric acid ($\geq 99\%$, crystalline) and L-ascorbic acid (reagent grade, crystalline), glucose, dopamine hydrochloride, epinephrine, glutaraldehyde (50%), polytetrafluoroethylene (PTFE, 60 wt%) were provided by Sigma-Aldrich. KH_2PO_4 and K_2HPO_4 were supplied by Emsure® and Sigma-Aldrich, respectively. H_2SO_4 (98%) was provided by AnalaR Normapur®. HClO_4 (60%) was purchased from VWR Chemicals. NaOH and 4-aminobenzoic acid were purchased from Merck. Ultrapure water was obtained from a Purelab Ultra Elga equipment with a resistivity of 18 M Ω cm.

Commercial gold thin-film interdigitated array microelectrodes (Au-IDA) were provided by Micrux Technologies. These devices consist of a working, a counter and a pseudoreference electrodes, all of them made of gold. The working electrode consist of two individual arrays of Au interdigitated electrodes. In the present manuscript, Au-IDA was used as the substrate for the hCNTs electrode because of its electrical properties and portability.

All electrochemical measurements were carried out in a BIOLOGIC SP-300 potentiostat.

2.2. Preparation of sensors

2.2.1. Electrochemical functionalization of hCNTs with 4-ABA

The hCNTs were functionalized from hCNT paste consisting of a mixture of hCNTs and PTFE as binder in a proportion 95:5 (w/w), respectively. Dried square-molded (1.5 cm²) paste containing 25 mg of this mixture was manually spread onto a graphite sheet collector to achieve an electrode with a thin uniform hCNTs coating. Then, potentiodynamic functionalization was achieved by submitting the prepared electrode to cyclic voltammetry (CV) in a 0.1 M HClO_4 solution containing 1 mM of 4-ABA, where the potential was scanned between 0.5 and 1.4 V at 10 mV s⁻¹ for 10 cycles obtaining the hCNTs-4ABA material [20]. A platinum (Pt) wire was used as counter electrode (CE) and an Ag/AgCl/ Cl^- electrode as reference electrode (RE).

The hCNTs-4ABA sample was dispersed in water (0.5 mg mL⁻¹) using an ultrasound probe for 10 min.

2.2.2. Preparation of modified electrodes

Au-IDA electrode was cleaned by cycling 10 times between -1.0 and 1.3 V vs gold pseudo-reference at 0.1 V s^{-1} in $0.05 \text{ M H}_2\text{SO}_4$. Then the Au-IDA was modified by casting hCNTs-4ABA dispersion on the microelectrodes array (hCNTs-4ABA/Au-IDA modified electrodes). The inset of Figure 1 shows the FESEM image of the hCNT-4ABA-modified Au-IDA surface. Finally, $1 \mu\text{L}$ of glutaraldehyde was added to improve the hCNTs-4ABA adhesion. The current was normalized to the deposited hCNTs mass (around $5 \mu\text{g}$) and it is referred to as current density (j).

2.3. Electrochemical methods

Figure 1 shows the two electrochemical cell configurations developed: beaker-cell (Figure 1A) and drop-cell (Figure 1B). In both designs, a three-electrode cell configuration was used, in which, the modified electrode (hCNT-4ABA/Au-IDA) was the WE, an Ag/AgCl/ Cl^- electrode was the RE and the CE was a Pt wire, which was wrapped around the RE.

Fig 1. Schematic illustration of the electrochemical cells showing the layout of the different electrodes; working (WE, hCNT-4ABA/Au-IDA), reference (RE; Ag/AgCl/ Cl^-), and counter (CE, Pt) electrodes: A) beaker-cell configuration with the vertical position of the WE and RE. B) Drop-cell configuration with the horizontal position of the WE. In both configurations the CE was wrapped around the RE. Inset: Electrode surface FE-SEM image showing the functionalized hCNTs.

Figure 1A shows the beaker-cell configuration consisting of a modified Au-IDA electrode (WE) in vertical position, inside 40 mL of phosphate buffer solution as electrolyte (PBS 0.25 M , pH 7.0). The RE and CE were also inside the solution. In this configuration, stirring was performed during the electrochemical measurement. Figure 1B exhibits the drop-cell configuration, which consists of WE in horizontal position. In this case, a drop of $40 \mu\text{L}$ is used as electrolyte. The beaker-cell configuration is useful for analyzing elevated AA and

UA concentrations in fluids like urine, which are easily collected. The beaker-cell also allows the use of more diluted samples, decreasing the matrix effects. However, the drop-cell allows the analysis of human fluids like serum or perspiration, which are difficult to collect in large volumes, and where the AA and UA concentrations are low.

The electrochemical behavior of the modified electrode was studied at different AA and UA concentrations and, subsequently, concentrated AA and UA stock solutions (100 mM) were prepared in PBS. Four different aliquots were added to the electrochemical cell (0.25 M PBS, pH 7.0) to reach the desired concentrations (50, 120, 420 and 600 μ M). Under this pH condition, and according to the pK_a of the two analytes (pK_a (AA) = 4.2 [45]; pK_a (UA) = 5.2 [46]), the ascorbate monoanion (HA^-) and urate (HU^-) were quantified. However, these analytes will be referred to as AA and UA throughout the manuscript.

AA and UA were quantified by chronoamperometry at 0.1 and 0.32 V, respectively, for concentrations between 0 and 1 mM of AA and UA, by adding seventeen aliquots of both analytes and by using the two cell configurations. All measurements were carried out in triplicate. Moreover, the limit of detection (LOD) was determined empirically, progressively measuring more diluted concentrations of the analyte. The LOD was the lowest concentration whose signal could be clearly distinguished from the blank. Moreover, the limit of quantification (LOQ) was calculated as 3.3 times the LOD ($LOQ = 3.3LOD$). Additionally, the relative standard deviations (RSD) were obtained for 3 replicates at the indicated concentration levels of the analytes [47].

For real samples analysis, filtered and diluted human perspiration and human urine were used and no other treatment was carried out. Diluted serum samples were also evaluated.

3. Results and discussion

3.1. hCNTs-4ABA characterization

The material was characterized using several techniques (XPS, TPD and TEM). The electrochemical behavior of the modified electrode (hCNTs-4ABA/Au-IDA) was also investigated by CV. The most relevant results are presented in Figure S1.

3.2. Oxidation of AA and UA using the beaker-cell configuration

3.2.1. Voltammetric behavior of the hCNTs-4ABA/Au-IDA electrode

Figure 2 presents the voltammetric and chronoamperometric behaviors of the hCNTs-4ABA/Au-IDA electrode for different AA and UA concentrations, using the beaker-cell configuration.

Fig 2. Electrochemical study of the hCNTs-4ABA/Au-IDA electrode towards AA and UA oxidation: A-B) Voltammograms for the hCNTs-4ABA/Au-IDA electrode in 0.25 M PBS, pH 7.0 at different: A) AA concentrations. B) UA concentrations. $v = 50 \text{ mV s}^{-1}$. C-D) Chronoamperometric curves for concentrations: 0, 0.65, 1.25, 2.5, 5, 10, 25, 50, 75, 100, 200, 250, 400, 500, 750 and 1000 μM of: C) AA at 0.1 V. Inset: enlargement at low concentrations. D) UA at 0.32 V. Inset: enlargement at low concentrations. $E_i = 0.0 \text{ V}$. E-F) Calibration curves obtained for: E) AA. F) UA. G-H) Chronoamperometric curves obtained for successive additions of aliquots to obtain: G) 30 μM AA, 420 μM UA, 5 mM glucose, 1 μM DP, 0.5 μM EP and 60 μM AA at $E_f = 0.1 \text{ V}$. H) 120 μM UA, 30 μM AA, 5 mM glucose, 1 μM DP, 0.5 μM EP and repeated twice at $E_f = 0.32 \text{ V}$. $E_i = 0.0 \text{ V}$. Beaker-cell configuration.

Figure 2A exhibits the voltammograms obtained with the hCNTs-4ABA/Au-IDA electrode for successive AA concentrations between 50 and 600 μM . As shown, an anodic peak related to the oxidation of AA appears at 0.085 V, whose intensity increases with the AA concentration. The voltammograms do not exhibit a cathodic peak, indicating the irreversibility of the process [48]. Figure 2B presents the voltammograms obtained with this electrode for concentrations of UA ranging from 50 to 600 μM . The oxidation peak is observed at 0.32 V, related with the quinone form [49], and its intensity increases with UA concentration.

Based on the literature, ascorbic acid oxidation at pH lower than 8 takes place by two successive one electron oxidation steps followed by a rapid dehydration, determining an overall irreversible process [48]. The oxidation peak belongs to the oxidation of hydroxy groups to carbonyl groups in furan ring of AA on hCNTs-4ABA. At pH 7, AA is negatively charged, being feasible the formation of hydrogen bonds between AA and hCNT-4ABA. Moreover, the comparison between the small cathodic current with the anodic current during UA oxidation suggest that the oxidation product of UA, is very unstable and undergoes a rapid chemical reaction. According to the literature, UA is oxidized to the quinoid form followed by a final decomposition reaction, leading allantoin as final product [49]. Then, the rich surface functional groups introduced in hCNTs-4ABA can interact with UA and AA by hydrogen bond or electrostatic force, improving the electrochemical detection of both compounds.

The analytical parameters obtained from the voltammograms analysis are summarized in Table 1, obtaining high correlation coefficients for both analytes and higher sensitivity for UA, albeit in the same order of magnitude as those obtained for AA.

3.2.2. Chronoamperometric study for the hCNTs-4ABA/Au-IDA electrode

3.2.2.1. Individual determination of AA and UA

From the analysis of the voltammograms, the individual quantification of AA and UA was performed by chronoamperometry at 0.1 and 0.32 V, respectively. The chronoamperometric curves for AA and UA concentrations between 0 and 1 mM are presented in Figures 2C and 2D, respectively. Figures 2E and 2F show the calibration curves obtained for both analytes.

All figures of merit calculated for the quantification of both analytes are summarized in Table 1. The sensitivities obtained with this technique are higher than those obtained through CV. Moreover, the LOD and LOQ are 20-fold lower. These values make this technique more suitable for the determination of trace levels of these analytes with this electrode.

3.2.2.2. Study of interferences

An amperometric study was undertaken for the minimum and maximum concentrations of AA and UA common in physiological fluids in order to evaluate the mutual interference. Moreover, other compounds that usually coexist in physiological fluids like serum were evaluated. For that purpose, the concentrations of UA analyzed were between 120 and 420 μM [1,2,50], AA between 30 and 90 μM [5,45,51], 5 mM glucose [52], 1 μM dopamine (DP) [53,54] and 0.5 μM epinephrine (EP) [55,56] were studied. Figures 2G and 2H display the chronoamperometric curves recorded during the interferences study.

Figure 2G shows that the successive additions of UA, glucose, DP and EP up to a concentration of 420 μM , 5 mM, 1 μM and 0.5 μM , respectively do not produce a significant change in the current obtained for the oxidation of 30 μM AA at 0.1 V. Whereas the successive additions of AA (30 μM and 60 μM) lead to an increase in the current density. Therefore, it can be concluded that UA, glucose, DP and EP do not interfere in the determination of AA at these concentrations. Figure 2H exhibits the chronoamperometric curve obtained for the addition of 120 μM UA, which produces an increase in the current

density. The successive addition of AA ($30\ \mu\text{M}$) again causes an increase in the current density and the additions of glucose ($5\ \text{mM}$), DP ($1\ \mu\text{M}$) and EP ($0.5\ \mu\text{M}$) at $0.32\ \text{V}$ do not produce any changes in the current density. After these additions, a UA concentration of $240\ \mu\text{M}$ was studied and, as expected, a higher current was observed. The subsequent increase in the concentration of AA up to $60\ \mu\text{M}$ increased the current. Then, as it can be observed in Figure 2H, neither glucose, DP, nor EP, at the concentration levels studied, interfered with the measurement of UA at $0.32\ \text{V}$. However, AA interferes at this potential. Nevertheless, the studied AA concentrations in Figure 2H were higher than the one found in the real samples, if the dilution is considered.

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Table 1. Figures of merit for the AA and UA determination by the hCNTs-4ABA/Au-IDA modified electrode. Beaker-cell and drop-cell configurations.

Parameter	Beaker-cell				Drop-cell			
	Cyclic voltammetry		Chronoamperometry		Chronoamperometry		Chronoamperometry	
	AA	UA	AA (0.1 V)	UA (0.32 V)	UA + AA (4 μ M AA) (0.32 V)	AA (0.1 V)	UA (0.32 V)	UA + AA (8 μ M AA) (0.32 V)
Sensitivity (A g ⁻¹ mM ⁻¹)	4.6 $\pm$ 0.8	6.4 $\pm$ 0.8	9.0 $\pm$ 0.4	8.8 $\pm$ 0.3	8.2 $\pm$ 0.6	2.00 $\pm$ 0.06	2.24 $\pm$ 0.08	2.3 $\pm$ 0.1
Intercept (A g ⁻¹)	0.2 $\pm$ 0.3	0.2 $\pm$ 0.8	(8 $\pm$ 6) $\cdot 10^{-2}$	(8 $\pm$ 6) $\cdot 10^{-2}$	0.2 $\pm$ 0.1	(-2 $\pm$ 6) $\cdot 10^{-3}$	(2 $\pm$ 2) $\cdot 10^{-2}$	(2 $\pm$ 2) $\cdot 10^{-2}$
r	0.994	0.997	0.998	0.996	0.993	0.999	0.998	0.997
N	5	5	14	14	14	12	14	14
Linear range (μ M)	0-600	0-600	0-500	0-500	0-500	0-250	0-500	0-500
LOD (μ M)	15	15	0.65	0.65	0.65	0.65	0.65	0.65
LOQ (μ M)	50	50	2.1	2.1	2.1	2.1	2.1	2.1
RSD (%) (n = 3; 50 μ M)	N.D. ^a	N.D. ^a	8	6	9	7	6	6

^aN.D.: Not determined.

The results reported here show that AA interferes in UA determination. In order to achieve UA determination in urine samples, a calibration curve of UA was performed in the presence of 4 μM AA, which was the final sample concentration after dilution procedure (Figure S2A, Table 1).

3.2.3. Determination of AA and UA in urine samples

Human urine was collected from five volunteers (3 males and 2 females). Only Subject V was diagnosed with kidney stones. Urine was filtered and diluted and no further treatment was carried out. The voltammograms obtained using the hCNTs-4ABA/Au-IDA electrode are presented in Figure S3, where the oxidation peak can be observed at 0.32V corresponding to UA oxidation.

UA was determined by chronoamperometry at 0.32 V and by using the external calibration curve performed in PBS (Figure 2F). The results obtained can be seen in Figure S3B.

The calculated concentrations of UA in real urine samples are shown in Table 2. It should be noted that the addition of known concentrations of UA (Figure S3B) allows for assessment of method trueness, whose recovery values should be between 80 and 120%, ensuring the quality of the measurement. As shown in Table 2, all the recoveries calculated are included in the established range, indicating the high trueness of the method. Table 2 exhibits the UA concentrations for all subjects, using different dilutions. It is noteworthy that in most samples the AA concentration was lower than the LOD or LOQ achieved with this method, due to the dilution (Table 1). This means that AA does not interfere with UA measurement, and the calibration curve obtained for UA using the beaker-cell configuration (Figure 2F, Table 1) is adequate. However, the AA concentration in the sample collected from Subject V, even when the dilution was 1:100, was higher than the LOQ ($\text{LOQ} = 2.1 \mu\text{M} < C_{\text{AA,V}} (\text{diluted } 1:100) = 3.99 \mu\text{M}$). For this reason, we used the calibration curve of UA performed with 4 μM AA, in order to determine the UA concentration in the urine sample V (Figure S2A and Table 1).

As shown in Table 2, the UA levels determined are close to the healthy concentration range (between 1.4 and 4.7 mM [57,58]). Additionally, the urine sample from Subject V was used to evaluate inter-sensor reproducibility using four different sensors, providing a RSD of 7% (Figure S4).

The use of the beaker-cell configuration does not allow for determination of these low AA concentrations in the diluted urine. Therefore, the drop-cell configuration was used.

Table 2. Determination of UA using beaker-cell configuration and AA using drop-cell configuration in urine samples.

Subject/ Analyte	Dilution	$C_{\text{sample}}^{a,b}$ (μM)	Spiked (mM)	Found ^c (mM)	Recovery (%) ^d
I	UA	1:50	0.1	0.160 ± 0.013	95 ± 12
			0.2	0.195 ± 0.011	83 ± 2
	AA	1:2	0.1	0.102 ± 0.011	93 ± 15
			0.2	0.204 ± 0.011	98 ± 6
II	UA	1:50	0.1	0.112 ± 0.011	91 ± 14
			0.2	0.185 ± 0.011	82 ± 15
	AA	1:2	0.1	0.177 ± 0.013	116 ± 15
			0.2	0.320 ± 0.013	112 ± 19
III	UA	1:100	0.1	0.098 ± 0.011	94 ± 5
			0.2	0.183 ± 0.011	90 ± 3
	AA	1:2	0.1	0.116 ± 0.011	82 ± 10
			0.2	0.220 ± 0.011	91 ± 11
IV	UA	1:100	0.1	0.10 ± 0.02	90 ± 8
			0.2	0.18 ± 0.02	85 ± 10
	AA	1:2	0.1	0.13 ± 0.02	103
			0.2	0.22 ± 0.02	98
V	UA	1:100	0.1	0.12 ± 0.02	97 ± 14
			0.2	0.21 ± 0.02	92 ± 13
	AA	1:2	0.1	0.274 ± 0.014	92 ± 2
			0.2	0.375 ± 0.014	94 ± 3

^a The concentration of the analyte in the sample taking into account the applied dilution.

^{b,c,d} Uncertainty as confidence interval.

3.3. Oxidation of AA and UA using the drop-cell configuration

3.3.1. Chronoamperometric study for hCNTs-4ABA/Au-IDA electrode

3.3.1.1. Individual determination of AA and UA

The chronoamperometric curves obtained for AA and UA in PBS at 0.1 and 0.32 V, respectively, are presented in Figures 3A and 3B, respectively, with calibration curves shown in Figures 3C (AA) and 3D (UA).

Fig 3. Electrochemical behavior of hCNTs-4ABA/Au-IDA electrode in PBS (0.25 M PBS, pH 7.0) towards AA and UA oxidation: A-B) chronoamperometric curves for concentrations: 0, 0.65, 1.25, 2.5, 5, 10, 25, 50, 75, 100, 200, 250, 400, 500, 750 and 1000 μ M of: A) AA at 0.1 V. Inset: enlargement at low concentrations. B) UA at 0.32 V. Inset: enlargement at low concentrations $E_i = 0.0$ V. C-D) Calibration curves obtained for: C) AA. D) UA. Drop-cell configuration.

The figures of merit obtained using the drop-cell configuration are presented in Table 1. Comparison between results obtained with both cell configurations shows that the sensitivities for beaker-cell are higher than that obtained with the drop cell, which could be mostly associated with the stirring performed during the analysis.

3.3.2. Determination of AA and UA in real samples

3.3.2.1. Urine samples

The voltammograms of the diluted urine sample obtained are similar to that obtained with the beaker-cell configuration (Figure S3C). The determination of AA was accomplished by using the external calibration curve performed in PBS (Figure 3C).

The use of the drop-cell configuration allows for dilution just twice with only 20 μ L of the urine sample. Chronoamperometry was performed at 0.1 V to quantify the AA in urine samples (Figure S3D). AA concentrations calculated for all subjects investigated can be found in Table 2. The average value of the AA excreted by urine has been established in 132 μ M [59] (based on a 1.5 L total urine volume in 24 hours). However, the concentration

of AA in urine samples reflects recent intakes of vitamin C [60], and it can fluctuate, which justifies the differences between the AA levels calculated above. Nevertheless, the AA concentration for Subject V is much higher than the others. This elevated AA concentration may be ascribed to the kidney stones diagnosed for Subject V [61].

As indicated in Table 2, the confidence intervals associated with the recovery calculated for the urine sample of Subject IV are missing, due to an insufficient amount of sample.

3.3.3. Perspiration samples

Perspiration samples were collected from Subjects I-V after a hard sport session. Perspiration samples were filtered and diluted 1.3 and 2 times for AA and UA measurement, respectively. Those dilutions were chosen due to the low concentration of AA and UA in perspiration samples and the difficulty of collecting this sort of fluids; moreover, dilution is mandatory for the method developed by adding the analyte aliquots after reaching a PBS steady current. The AA and UA were quantified in perspiration samples by CV and chronoamperometry (Figure S5). The concentrations of AA and UA were calculated by using the external calibration curves presented in Figures 3C and 3D and the results can be found in Table 3.

On average, the AA concentration in urine was higher than in perspiration. The AA levels calculated for Subjects I-III proved lower than the LOD or LOQ of the method. For instance, the j-t curve registered at 0.1 V showed no differences after the addition of the perspiration sample collected from Subject I. On the other hand, the AA concentration in perspiration samples collected from Subjects II-III was high enough to observe an increase in the signal registered on the j-t curve. However, the AA concentration was lower than the LOQ of the method, and, consequently, the AA concentration could not be accurately determined in these two perspiration samples.

The healthy concentrations levels of AA in perspiration have been established between 0.11 and 36 μM [62], thus the two successfully determined AA values are included in the fixed range.

Table 3. Determination of AA and UA in perspiration and serum samples with the drop-cell configuration.

Sample	Subject/ Analyte	Dilution	C _{sample} ^{a,b} (μ M)	Spiked (mM)	Found ^c (mM)	Recovery (%) ^d
PERSPIRATION	I	AA 1:1.3	< LOD	0.1	0.11 $\pm$ 0.02	97 $\pm$ 5
				0.2	0.18 $\pm$ 0.02	88 $\pm$ 5
		UA 1:2	27 $\pm$ 13	0.1	0.125 $\pm$ 0.014	110 $\pm$ 13
				0.2	0.197 $\pm$ 0.015	93 $\pm$ 13
	II	AA 1:1.3	< LOQ	0.1	0.102 $\pm$ 0.006	102 $\pm$ 25
				0.2	0.193 $\pm$ 0.006	96 $\pm$ 12
		UA 1:2	13 $\pm$ 13	0.1	0.107 $\pm$ 0.013	101 $\pm$ 22
				0.2	0.208 $\pm$ 0.013	101 $\pm$ 11
	III	AA 1:1.3	< LOQ	0.1	0.121 $\pm$ 0.006	120 $\pm$ 5
				0.2	0.225 $\pm$ 0.008	112 $\pm$ 2
		UA 1:2	< LOQ	0.1	0.106 $\pm$ 0.013	107 $\pm$ 8
				0.2	0.209 $\pm$ 0.013	105 $\pm$ 5
	IV	AA 1:1.3	3 $\pm$ 6	0.1	0.116 $\pm$ 0.007	114 $\pm$ 8
				0.2	0.223 $\pm$ 0.011	110 $\pm$ 4
		UA 1:2	< LOQ	0.1	0.10 $\pm$ 0.02	103 $\pm$ 4
				0.2	0.20 $\pm$ 0.02	100 $\pm$ 1
	V	AA 1:1.3	4 $\pm$ 10	0.1	0.090 $\pm$ 0.007	89
				0.2	0.206 $\pm$ 0.008	102
		UA 1:2	39 $\pm$ 13	0.1	0.124 $\pm$ 0.015	90 $\pm$ 6
				0.2	0.22 $\pm$ 0.02	94 $\pm$ 8
SERUM	II	AA 1:2	40 $\pm$ 6	0.1	0.107 $\pm$ 0.006	89 $\pm$ 12
				0.2	0.259 $\pm$ 0.006	117 $\pm$ 14
		UA 1:5	281 $\pm$ 13	0.1	0.163 $\pm$ 0.013	105 $\pm$ 2
				0.2	0.259 $\pm$ 0.006	117 $\pm$ 14
	VI	AA 1:2	18 $\pm$ 6	0.1	0.104 $\pm$ 0.006	95 $\pm$ 17
				0.2	0.193 $\pm$ 0.007	92 $\pm$ 26
		UA 1:2.25	202 $\pm$ 13	0.1	0.155 $\pm$ 0.013	84 $\pm$ 6
				0.2	0.358 $\pm$ 0.014	110 $\pm$ 22

^a The concentration of the analyte in the sample taking into account the applied dilution.^{b,c,d} Uncertainty as confidence interval.

However, when UA analysis was performed, the AA concentration was lower than the LOD or LOQ due to the dilution of the perspiration sample. As a result, the calibration curve obtained for UA using the drop-cell (Figure 3D) was employed to determine the UA concentration.

By contrast, the UA concentration in perspiration samples collected from Subjects III-IV displayed a noticeable increase in the current density recorded at 0.32 V on sample addition but the concentration was lower than the LOQ

The trueness of the method was evaluated by adding two known concentrations of AA and UA on a perspiration matrix. As shown in Table 3, all the recoveries calculated, for both AA and UA, are included in the established range between 80 and 120%. As a result, we can conclude that no significant matrix effect was detected with the samples investigated allowing the use of an external calibration curve.

The concentrations of UA determined in perspiration samples from Subjects I-V are included in the established range for healthy people or near to it (between 4.2 μM and 4.8 mM) [62].

The possible correlation between the AA and UA concentrations found in urine and perspiration samples was evaluated. The results can be seen in Figure S6.

3.3.4. Serum samples

Serum from blood samples was collected from two healthy subjects, Subject II and VI. The AA and UA were analyzed by chronoamperometry of the diluted serum sample (1:2) at 0.1 and 0.32 V, respectively, and by using the drop-cell configuration (Figure S7).

Table 3 shows the values obtained in the quantification of both analytes using the external calibration curves presented in Figures 3C and S2B. Figure S2B was employed because AA concentration is higher in serum samples than in other fluids and it is not possible to avoid the AA interference just by dilution. This was solved using the calibration curve performed in the presence of AA (Table 1). The AA concentration levels for healthy people in serum are between 30 and 90 μM [5,51]. Thus, the sample collected from Subject II is within the established range, while AA concentration calculated in the serum from Subject VI may indicate marginal deficiency [5].

Normal levels of UA in serum are between 120 and 450 μM [6,63], thus Table 3 shows that both serum samples analyzed have a UA concentration included within this range.

All the calculated recoveries, for both AA and UA, are included in the acceptability range between 80 and 120%. Thus, it can be concluded that the developed method is able to determine AA and UA in serum samples by using an external calibration curve. In addition, to demonstrate that the characteristics of the method are suitable for the quantification of UA in serum matrixes, the UA concentration in the serum sample collected from Subject VI was analyzed by a certified laboratory. An AU5800 analyzer in conjunction with the uricase-peroxidase method was used, which is the most common method for UA determination in blood [64]. The concentration of UA from this analysis was 3.51 mg dL⁻¹.

Comparing this value with the one obtained in this work, it can be concluded that there are no significant differences between these two results ($C_{UA} = 3.4 \pm 0.2 \text{ mg dL}^{-1}$; 96.9 % recovery). As a result of the adequate trueness and precision of the method, we can conclude that the method provides remarkably accurate results.

Finally, Table S1 summarizes some of the methods developed for the quantification of AA, UA or both. To the best of our knowledge, this is the first reported portable sensor able to determine AA and UA on three different human fluids (urine, perspiration and serum) without matrix effects after dilution, allowing the sensitive determination of the levels of AA and UA by using an external calibration method.

Conclusions

A new portable electrochemical sensor based on 4-aminobenzoic acid functionalized herringbone carbon nanotubes using an array of gold interdigitated microelectrodes (hCNTs-4ABA/Au-IDA) has been developed for AA and UA quantification in physiological fluids. AA and UA were quantified by chronoamperometry at 0.1 and 0.32 V, respectively, and two electrochemical cell configurations were used: drop-cell and beaker-cell, each one with different design and function. Separate quantification of AA and UA was successfully achieved, with LODs and LOQs of 0.65 μM and 2.1 μM , respectively. The simultaneous quantification of AA and UA has been studied without observable interference of UA in the AA measurements. However, if AA is in the mixture the UA concentration results may vary depending on the AA concentration. No interferences of glucose, DP and EP were observed, confirming the high specificity of the sensor towards AA and UA. The method has been successfully applied to filtered and diluted urine and perspiration samples and diluted serum samples. The AA and UA concentrations were calculated by using an external calibration curve for each analyte, thus saving time and sample.

These data indicate that the present portable electrochemical sensor is viable, and potentially a powerful and promising alternative for the quantification of AA and UA levels in various human fluids.

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Video showing the uric acid quantification in a serum sample and recovery determination

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Highlights

- electrochemical and portable sensor based on 4-aminobenzoic acid-herringbone carbon nanotubes
- simultaneous quantification of ascorbic acid and uric acid in physiological fluids has been done
- The sensor has been successfully used on human urine, perspiration and serum, without matrix effects