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Grado en Química

Determination of homogentisic acid using HPLC with amperometric detection at carbon paste electrodes

Autor: María Lozano Alonso

Tutores: Hana Dejmková

María del Sol Vega Alegre

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María Lozano Alonso

Supervisors: H. Dejmková, M. Vega.

Charles University, Faculty of Science, Department of Analytical Chemistry, University Research Centre Supramolecular Chemistry, UNESCO Laboratory of Environmental Electrochemistry, Albertov 6, CZ-128 43 Prague 2, Czech Republic, dejmkova@natur.cuni.cz

Abstract

Homogentisic acid is an intermediate of the metabolic breakdown of tyrosine and phenylalanine; its accumulation in body fluids suggests the break in the metabolic pathway of these compounds and related health-threatening complications. In our work, a determination method for this compound was developed using HPLC with amperometric detection on glassy carbon paste electrode. Optimum conditions were found, considering both electrode response and chromatographic parameters. Separation was performed on Kromasil 100 C18, 250 x 4.6 mm (5 µm) column using gradient elution; mobile phase consisted of acidic buffer and methanol, with methanol content changing from 5% to 30% in 10 min. Repeatability of the measurements and linearity of the electrode response were confirmed. Applicability of the method was proved by the determination in spiked urine samples.

Resumen

El ácido homogentísico es un intermediario de la descomposición metabólica de la tirosina y la fenilalanina; su acumulación en los fluidos corporales sugiere una ruptura en la vía metabólica de estos compuestos y da lugar a complicaciones en la salud. En este trabajo se ha puesto a punto un método para la determinación de este compuesto usando HPLC con detección amperométrica con electrodo de pasta de carbono vitrificado. En la elección de las condiciones óptimas se tuvo en cuenta tanto la respuesta del electrodo como los parámetros cromatográficos. La separación se llevó a cabo con una columna Kromasil 100 C18, 250 x 4,6 mm (5 µm) usando gradiente de elución; la fase móvil consistió en una disolución tampón ácida y metanol, cambiando el contenido de metanol desde 5% a 30% en 10 min. La repetibilidad de la medición y la linealidad de la respuesta del electrodo fueron validadas. La aplicabilidad del método se comprobó mediante la determinación en muestras de orina dopadas con el analito.

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1. ABREVIATIONS

AD	Amperometric detector
AKU	Alkaptonuria
C16	Hexadecylsilane
C18	Octadecylsilane
CE	Capillary electrophoresis
CPE	Carbon paste electrode
DAD	Diode array detector
E	Potential
EC	Electrochemical detector
ESI	Electrospray ionization detector
GC	Gas chromotagraphy
HGA	Homogentisic acid
HGD	Homogentisate 1,2-dioxygenase
HPLC	High performance liquid chromatography
I_a	Anodic current
I_c	Cathodic current
IC	Ion chromatography
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry detector
PVDF	Polyvinylidene difluoride
SD	Standard deviation
TFA	Trifluoroacetic acid
UV	Ultraviolet
λ	Wavelength

2. INTRODUCTION

Homogentisic acid or 2,5-dihydroxyphenylacetic acid (HGA) is a small molecule which can be found in the human body. It is an intermediate of the metabolic breakdown of tyrosine and phenylalanine [1]. HGA structure is shown in Figure 1 and its physical and chemical properties may be found in Table 1 [2,3].

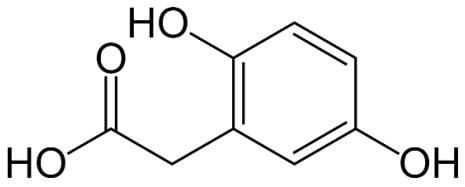


Figure 1. *Structure of homogentisic acid*

Table 1. *Physical and chemical properties of HGA*

Name (IUPAC)	2,5-Dihydroxy-phenylacetic acid
Common name	Homogentisic acid
Molecular formula	$C_8H_8O_4$
Molecular weight	168.167 g/mol
Chemical abstracts service (C.A.S.) number	451-13-8
Melting point	153ºC
Boiling point	452ºC
Storage temperature	2-8ºC
Water solubility	8.1 mg/mL
Stability	Stable in strongly acidic media. Oxidized by dissolved oxygen in alkaline media.
Acidity constant (pk_a)	4.40

Tyrosine is a non-essential amino acid and can, therefore, be synthesized by the organism from amino acids obtained through the diet. In the case of tyrosine, the essential amino acid source is phenylalanine. Tyrosine and phenylalanine are very important in our organism because they produce, among other things, neurotransmitters such as dopamine and noradrenaline. In other words, these amino acids play an important role in the regulation of the state of alert and vitality, mood regulation or promotion of correct nervous system functioning. The role of tyrosine and phenylalanine does not cease in brain but continues with the synthesis of melanin, related to the skin pigmentation [4,5].

Under normal conditions, tyrosine is metabolized into homogentisic acid which is oxidized to 4-maleylacetoacetic acid by homogentisate 1,2-dioxygenase (HGD). This is the reason of the small level of HGA in urine, sometimes undetectable in healthy humans [6,7]. When this metabolic pathway is not completed it results in an illness called alkaptonuria (AKU), which is a genetic anomaly that makes the human body unable to break down the tyrosine amino acid. Consequently, a lack of HGD is produced, which leads to an accumulation of HGA in connective tissues, especially cartilage [8]. The high concentration of the acid in the blood cause to be excreted in urine and that is why blood and urine are the biofluid locations to analyze HGA [1].

AKU is a hereditary disease that appears because of an autosomal recessive condition. Almost the 100% of AKU patients have mutations in HGD gene located in the long chromosome arm (3q21-23) and they show a great allelic variety [9]. AKU is a rare disease only suffered by 1 in 100 000 persons [10], but it is endemic in Slovakia, Czech Republic, and Dominican Republic [11].

The first symptom to detect alkaptonuria, which is present from birth, is dark color of urine after adding alkali or being exposed to air for several hours (due to the oxidation of HGA). Since the color change takes time, it often goes unnoticed [12]. The diagnosis is often given about the age of 30, when severe symptoms usually start. Affected individuals develop ochronosis (pigmentation of connective tissue) and joints degenerative arthrosis. When the patient reaches the age of 40, a scleral pigmentation

begins, followed by a discoloration of ear cartilage. Furthermore, paleness of the forehead, cheeks, axilla, palms, nails, and soles may be observed. In addition, at this age joints arthrosis may start, for example in knees, intervertebral spine and shoulders. The disease might get as severe as to require a total joint replacement. Aortic or mitral valvulitis, calcification of coronary arteries and atherosclerotic plaques are seen after the age of 50 years [6, 12].

AKU diagnosis may be made through high HGA concentration excreted in urine [11], osteoarthrosis early developing, dark color of the urine when exposed to air or family history of AKU diagnosis [8].

Despite of the severity of the disease there is not specific treatment. Nevertheless, an early diagnosis as well as the application of new therapeutic options can greatly improve the life quality of patients suffering from AKU [13].

Thereby, there have been several investigations to determinate HGA concentration in urine with different analytical methods (cf. Table 2).

Every method for HGA quantification in urine has its advantages and disadvantages. For example, in terms of sensitivity, the one that has the lowest is ion chromatography (IC). Liquid chromatography -mass spectrometry (LC-MS) shows good precision and trueness [10]. Capillary electrophoresis (CE) has also distinctive advantages, in terms of higher column efficiency and low sample volume requirement. Comparing ultra-violet (UV) detector to amperometric (electrochemical) detector (AD or EC), the latter is more sensitive, selective and cheap than laser-induced fluorescence detector which makes EC detection very popular for analysis. [14].

On the other hand, gas chromatography with mass spectrometry detector(GC-MS) and high performance liquid chromatography (HPLC) exhibit high sensitivity but they require the use of heavy equipment. The weak point of GC-MS is that it is very time-consuming when it comes to sample preparation procedures such as extraction and derivatization to increase the volatility. Furthermore, HPLC has a high reproducibility and the time

invested on sample preparation is shorter. Considering all the above, it can be concluded that HPLC is the most specific separation method but it the costs of equipment and maintenance are relatively high.

Table 2. *Selected methods for HGA determination.*

METHOD	MEDIUM (pH)	DETECTION SYSTEM	LOD (µg/L)	MATRIX	REFERENCES
HPLC-UV	acid	UV-vis	26 mg/kg	Honey	[15]
IC	6		15 mg/kg	Honey	[15]
HPLC-MS		Mass spectrometer	10 µg/L	Urine	[6]
HPLC-DAD	8	Diode array detector	60 µg/L	Standard solution	[16]
HLC-DAD-ESI-MS		Diode array detection and electrospray ionization tandem mass spectrometry	160 µg/L	Standard solution	[17]
GC-MS		Mass spectrometry	3.82 µg/L	Urine	[18]
CE	7	Amperometric detection	3.33 µg/L	Urine	[14]

3. OBJECTIVES

The aim of this work is to develop a fast, inexpensive, accurate, and sensitive method for HGA quantification in the urine of AKU patients.

An analytical method based on HPLC with amperometric detection using a carbon paste electrode will be optimized. For this purpose, the redox behavior of HGA on carbon paste electrodes will be studied using cyclic voltammetry in order to prove the reversibility of HGA oxidation on the electrode surface as the amperometric detector used in HPLC must show a fast and reproducible response to the analyte.

The method will be validated evaluating performance characteristics such as repeatability (precision), linearity, sensitivity and accuracy (proved by the absence of matrix effects).

4. EXPERIMENTAL PROCEDURE

4.1. Preparation of solutions

Accurately weighted HGA (4.2 mg) was transferred into a 25 mL measuring flask. It was dissolved in distilled water to get the stock solution with a final concentration of $1 \cdot 10^{-3}$ M.
The Britton-Robinson buffer was used to adjust the pH of the solutions to the desired value. It consists of a mixture of orthoboric acid 0.04 M, orthophosphoric acid 0.04 M and acetic acid 0.04 M. In experiments requiring a controlled pH, the buffer pH was adjusted by addition of the necessary amount of 0.02 M sodium hydroxide and then mixed with the sample.

4.2. CYCLIC VOLTAMMETRY

4.2.1. Apparatus

Voltammetric measurements were carried out using a computer-controlled Eco-Tribo Polarograph with Polar Pro software, version 5.1 for Windows 95/98/Me/2000/XP.

A three-electrode electrochemical cell was used: AgCl/Ag, KCl (saturated) and Pt were used as reference and auxiliary electrodes, respectively. A carbon paste electrode (CPE) of 2 mm inner diameter was used as working electrode. This electrode was prepared mixing 250 mg of glassy carbon spherical powder (10-20 µm, type 2, Alfa Aesar) and 100 µl of mineral oil. These components were crushed in a mortar until they were homogeneously mixed. It was obtained a pasty mixture which was introduced in the electrode body with a spatula.

4.2.2. Procedure

Cyclic voltammograms were recorded on solutions contained in the voltammetric vessel prepared by mixing 0.5 mL of the $1 \cdot 10^{-3}$ M HGA stock solution and 4.5 mL of the buffer at the selected pH value (final HGA concentration $1 \cdot 10^{-4}$ M).

After immersing the three electrodes in the solution, the cyclic voltammogram was recorded from an initial potential of -200 mV to a final potential of +1500 mV at a scan rate of 100 mV/s and then in the reverse direction. Voltammograms were recorded in triplicate. Peak current and peak potential were recorded from each voltammogram and mean results are shown.
The carbon paste electrode was cleaned before each measurement so the reactions can occur in a renewed surface every time. The carbon paste electrode was cleaned by wiping the surface with a wet filtration paper. Another precaution to have is to make sure that there is no bubble on the electrode surface for the reaction to occur on the whole area.

4.3. HPLC

4.3.1. Instruments and chromatographic conditions

HPLC analysis was carried out using an HPLC instrument consisting of a vacuum degasser, ternary pump with gradient elution (Ecom, CR) and manual injection valve. Two differet detectors were used: ultraviolet (UV) detector with deuterium lamp Sapphire 600 (Ecom, CR) and amperometric detector ADLC 2 (Laboratorni Pristroje, Praha) using the same working electrode as in cyclic voltammetric measurements.

The selection of the column was based on the results of HGA determination in previous investigations. It is shown in Table 3 that the characteristics of the columns are similar in every study. Therefore, a Kromasil 100 C18 reverse phase column with dimensions 250 x 4.6 mm and 5 µm particle size was selected in this study.

Table 3. *Chromatographic conditions for HGA determination in other studies.*

Mobile phase	**A**	H_2SO_4 $5 \cdot 10^{-3}$ mol/L		Acetonitrile (40%) and acetic acid (0.05%)		TFA (0.01%)		TFA (0.1%)		
	B	MeOH		Acetic acid (0.05%)		Acetonitrile		Acetonitrile		
Gradient elution	**Time (min)**	2	20	38	5	5	35	5	20	60
	A (%)	90	10	100	0	90	10	95	60	25
	B(%)	70	30	0	100	47	53	5	40	75
Flow rate (mL/min)		1.2		0.2		0.6		1		
λ (nm)		291						205		
Column		C18 250 mm x 4.6 mm 5 µm particle size		C16 100 mm x 2.1 mm		C18 250 mm x 4.6 mm 5 µm particle size		C18 250 mm x 4.6 mm 5 µm particle size		
Ref		[15]		[6]		[16]		[17]		

The maximum absorbance for HGA is 290 nm, which was determined by UV-vis spectrophotometry; this value was set on the HPLC equipment for all the measurements made with UV detector.

Room temperature was used as the column temperature. The mobile phase consisted of methanol (MeOH) and a buffer composed by 0.05 M phosphoric acid and 0.05 M acetic acid and subsequent addition of 0.02 M sodium hydroxide to adjust the pH when necessary. Flow rate was 1.0 ml/ min.

4.3.2. HPLC method

To find the optimum chromatographic conditions for HGA quantification, a series of experiments were made using two different detectors: UV spectrophotometric and amperometric with carbon paste electrode.

In order to acquire a good separation and maximum response with amperometric detection, the effects of mobile phase pH and electroanalytical detector potential on the peak area were studied with HGA stock solution diluted to $1 \cdot 10^{-4}$ M. The measurements were made three times for each potential at different pH values. Isocratic elution was used in this optimization experiment, even though gradient elution was used for real samples. Optimized HPLC conditions selected for further measurements are:

- Electroanalytical detector potential: 0.6 V
- Mobile phase: Isocratic gradient with 20% of MeOH and 80% of buffer (0.005 M H_3PO_4 and 0.05 M CH_3COOH) at pH 2.5

4.3.3. Urine sample preparation

Urine samples were obtained from a healthy student at Charles University in Prague, and then immediately analyzed. Urine samples were filtered through 0.45 µm PVDF filters.

The optimum gradient elution conditions for HGA separation in urine samples were found and are shown in Table 4. The content of methanol is increased from 5% to 30% in 10 min; in the next minute it is increased to 80% and kept there for 2 min in order to wash compounds with high retention time out the column. Then, initial conditions are restored in one minute.

Despite the cleaning steps, it is necessary to wash the column with 100% of MeOH every day to reduce matrix effects.

Each urine sample was measured three times and mean results are given.

Subsequently, urine samples were spiked with known amounts of the analyte ranging from $1 \cdot 10^{-4}$ M to $5 \cdot 10^{-6}$ M HGA and measured in the same experimental conditions.

Table 4. *Mobile phase composition in the HPLC gradient elution mode*

Time (min)	Solvent A (MeOH) (%)	Solvent B (0.05 M H_3PO_4 and 0.05 M CH_3COOH at pH 2.5) (%)
0	5	95
10	30	70
11	80	20
13	80	20
14	5	95

5. RESULTS AND DISCUSSION

5.1. CYCLIC VOLTAMMETRY

Cyclic voltammetry (CV) is an electroanalytical technique that provides information on the reaction mechanism taking place on the electrode surface such as oxidation and reduction reactions, adsorption processes, coupled reactions or nucleation [24]. It also helps to the determination of thermodynamic and kinetic parameters of the redox processes. CV involves sweeping the electrode potential from an initial potential to a final potential at a constant scan rate, *v*, and then the sweep is reversed, usually at the same scan rate, while the solution stays unstirred. The current intensity, *I*, is recorded as a function of the sweep potential, *E*. Anodic and cathodic current registered correspond to oxidation and reduction reactions taking place on the electrode surface.

To investigate the reversibility of the redox reaction of HGA on the glassy carbon paste electrode, cyclic voltammograms at different sweep rates were recorded on a solution containing 0.05 M HGA at pH 2.5. [24]. Figure 2 shows a selection of cyclic voltammograms recorded.

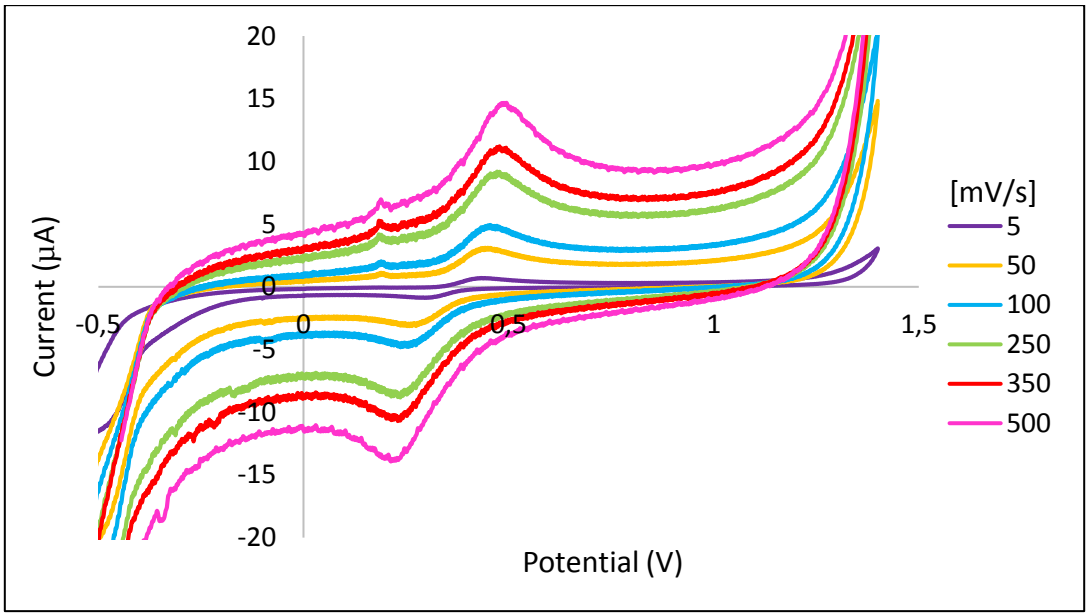


Figure 2. *Cyclic voltammograms obtained at pH 2.5 for $1 \cdot 10^{-4}$ M HGA recorded at different sweep rates.*

The dependence of current and potential with the scan rate is depicted in Figure 3. The peak potential is independent of scan rate up to 350 mV/s, but shifts negatively at higher scan rates indicating that the redox reaction of HGA at the CPE is reversible at low sweep rates, but turns in quasi-reversible and then irreversible as *v* increases.

Anodic peak current increases linearly with $v^{1/2}$ up to about 300 mV/s (see Figure 4), thus confirming the reversibility of the redox reaction of HGA on a CPE electrode at low sweep rates. From 300 mV/s *I* continues to increase with $v^{1/2}$, but not linearly, thus indicating that the mass transfer rate becomes slower than the electron transfer rate at high *v*, thus resulting in an increase of the irreversible nature of the electrodic process.

If peak current increases linearly with *v*, the electrodic reaction is adsorption controlled. If peak current depends linearly with $v^{1/2}$, the electrodic process is diffusion controlled. Figure 3 and Figure 4 show that the HGA redox reaction is predominantly controlled by diffusion at sweep rates up to 300 mV/s.

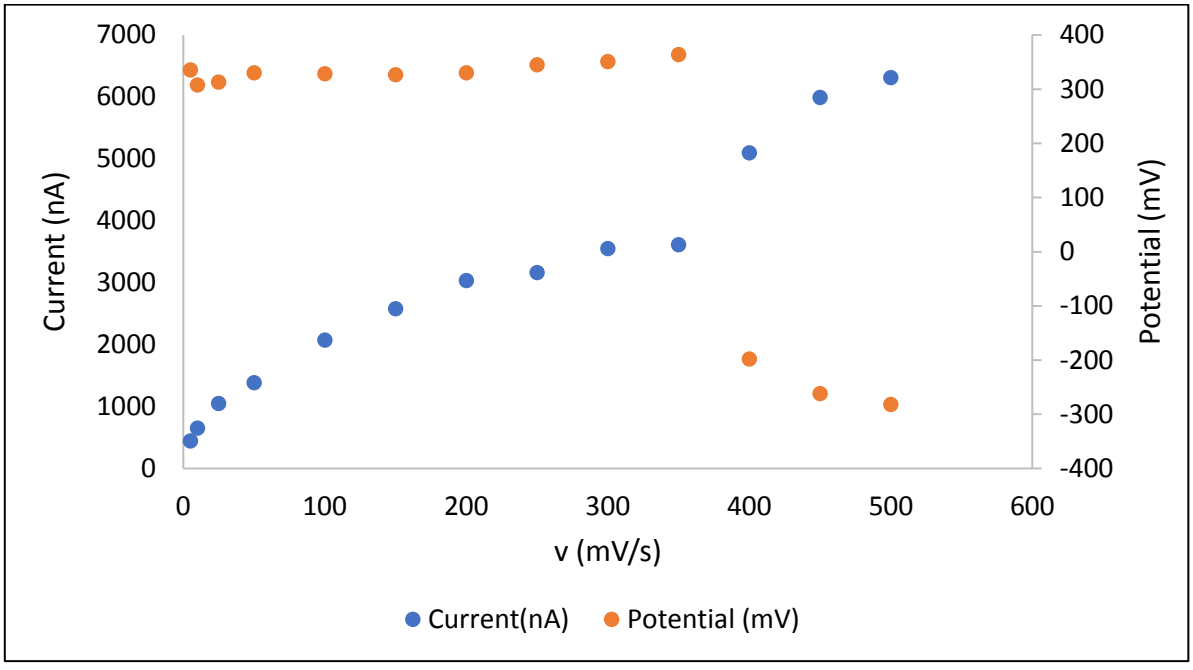


Figure 3. *Variation of anodic peak current and potential of 0.05 M HGA at pH 2.5 as a function of CV scan rate.*

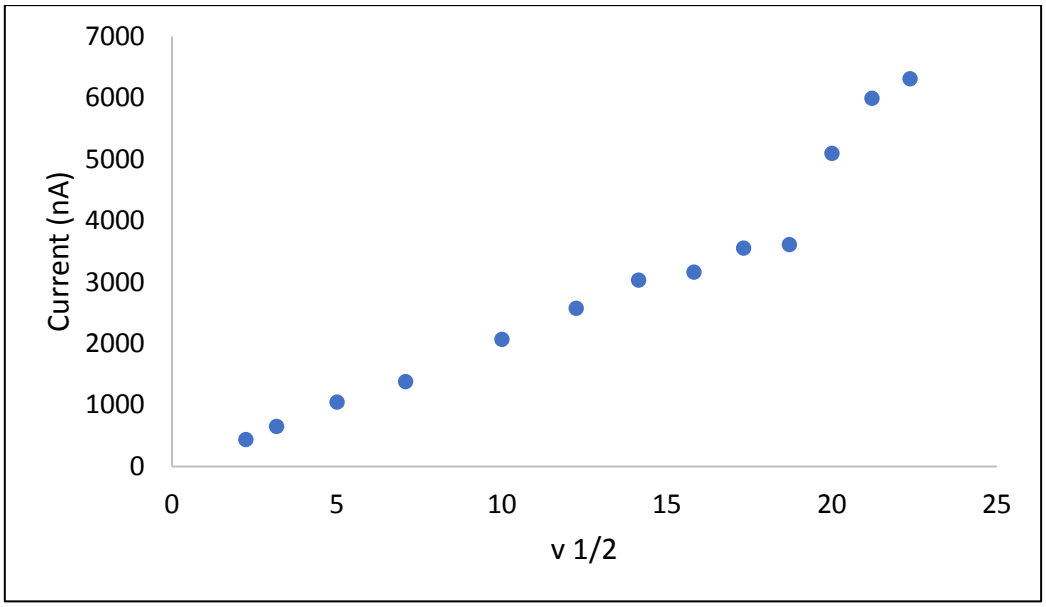


Figure 4. *Anodic peak current variation of 0.05 M HGA in Britton-Robinson buffer at pH 2.5 as a function of the square root of scan rate.*

To further assess this conclusion, a plot of log I vs. log v was obtained (Figure 5) to determine the slope of the curve which would be the parameter that defines how much of the reaction is controlled by diffusion or by adsorption according to the following equalities:

$$I = k\, v^{a}$$

$$\log I = \log k + a \log v$$

Where *k* is a constant, *a* is the slope of the linear curve, that must be ½ for diffusion controlled redox reactions, *v* is scan rate and *I* is the anodic peak current.

A slope of *a*=0.50 has been obtained, which confirms that the reaction is more diffusion controlled than adsorption controlled.

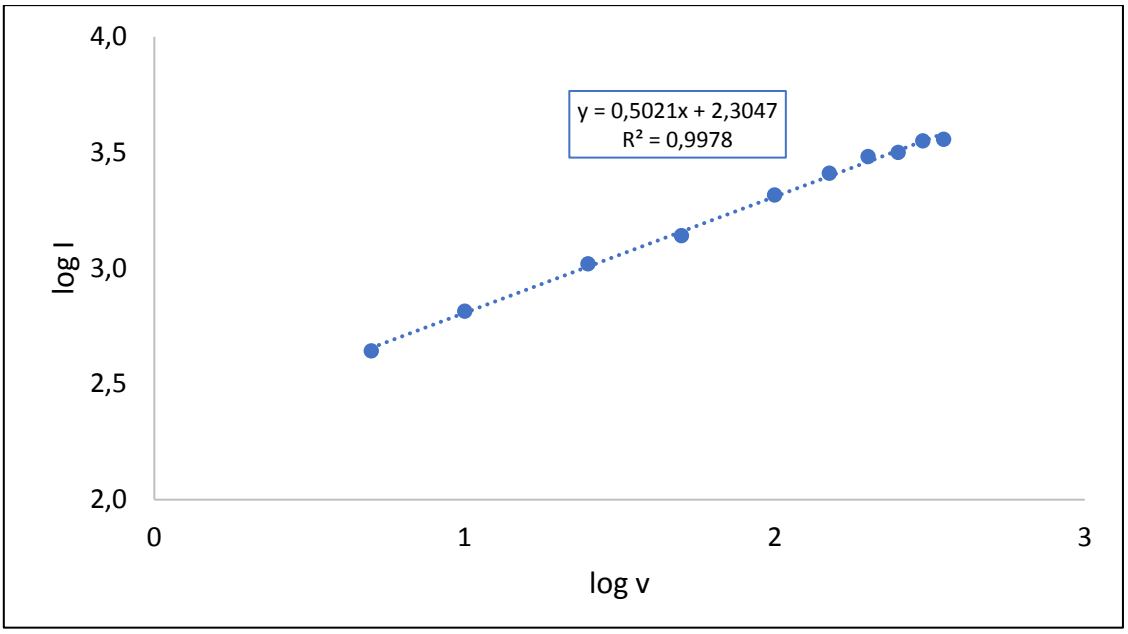


Figure 5. Logarithmic relationship between a*nodic peak current and scan rate of 0.05 M HGA at pH 2.5.*

If the electrodic reaction of HGA is reversible, the current of cathodic and anodic peaks must be equal, and therefore the I_a/I_c ratio tends to 1. Also, the difference between the anodic and cathodic peak potentials should follow the equation:

$$E_a - E_c = 2.303 \frac{RT}{nF}$$

where R is the gas constant, F is the Faraday constant, n is the number of electrons exchanged and T is the temperature (in K). At 298 K, the equation can be simplified:

$$E_a - E_c = \frac{59}{n} \text{ mV}$$

In the above equations and onwards, *a* and *c* subscripts symbolize anodic and cathodic peaks, respectively.

The difference between the peak potentials becomes larger as the irreversibility increases.

Table 5 . *Peak current and potential registered by CV as a function of the sweep rate.*

v (mV/s)	I_a (nA)	I_c (nA)	I_a/I_c	E_a (mV)	E_c (mV)	E_a-E_c (mV)	n
5	440	-500	0.88	436.4	335.6	100.8	0.59
10	651	-667	0.98	455.1	307.6	147.5	0.40
25	1046	-949	1.10	455.1	313.2	141.9	0.42
50	1382	-1387	1.00	468.2	330.0	138.2	0.43
100	2070	-1723	1.20	415.9	328.1	87.8	0.67
150	2576	-2211	1.17	470.0	326.2	143.8	0.41
200	3033	-3001	1.01	479.4	330.0	149.4	0.39
250	3161	-3485	0.91	451.4	344.9	106.4	0.55
300	3551	-3653	0.97	466.3	350.5	115.8	0.51
350	3610	-4339	0.83	460.7	363.6	97.1	0.61

As Table 5 shows, I_a/I_c ratio is close to 1 in the *v* range 5 - 350 mV/s (average value 1.00). The average distance between the anodic and cathodic peaks is 123 mV, which is compatible with a reversible process exchanging 2 electrons (average *n* is 0.50). Departure from these values was observed at higher scan rates, indicating a lost of reversibility as the mass transfer becomes the limiting step.

The conclusion drawn from all these data is that the electrodic reaction of HGA at the CPE is reversible at low sweep rates, becoming quasi-reversible and then irreversible at higher sweep rate. Thus, 100 mV/s has been chosen for further cyclic voltammetric measurements for the redox reaction to be reversible.

At that point, it has been required to investigate the effect of pH on the HGA redox behavior meaning to establish the pH range in which the HGA oxidation occurs.

Cyclic voltammograms (CV's) registered at even pH values are shown in Figure 6. No peaks were registered at pH>10. Table 6 displays some experimental parameters derived from the CV's.

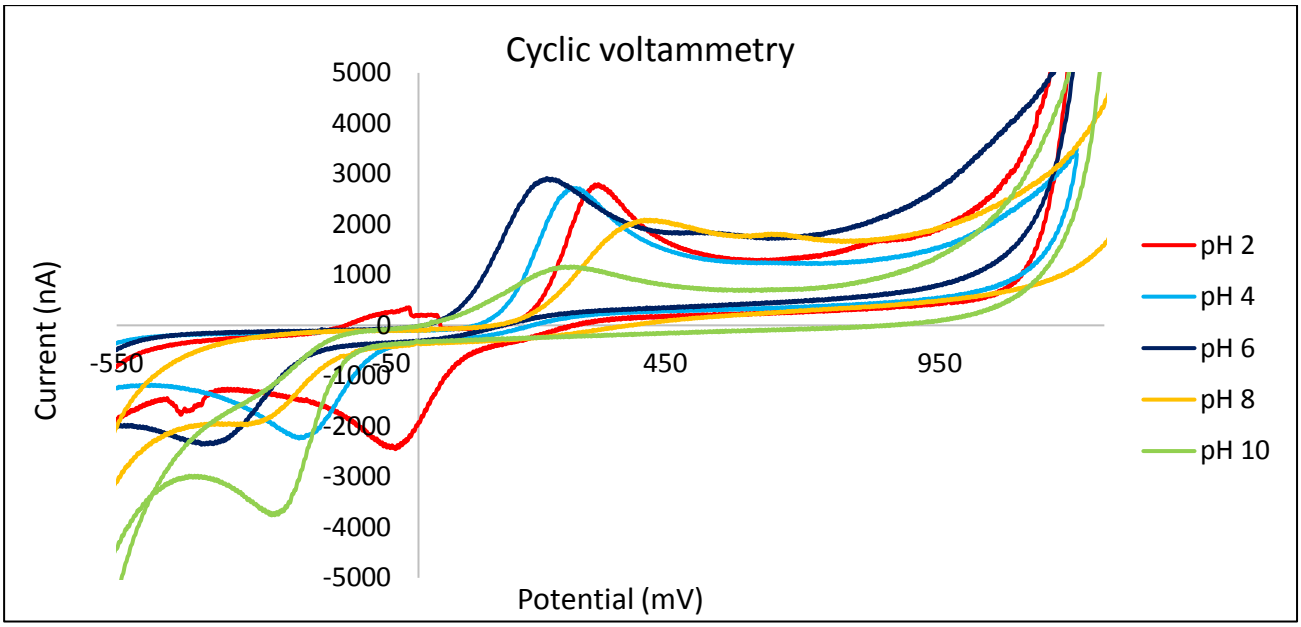


Figure 6. Cyclic v*oltammograms obtained at 100 mV/s sweep rate for 1·10^{-4} M HGA in Britton- Robinson buffer recorded at different pH values.*

Table 6 . *Peak currents and potentials registered by CV as a function of the pH. Peak data shown are the average of three measurements*

pH	I_a (nA)	I_c (nA)	I_a/I_c	E_a (mV)	E_c (mV)	E_a-E_c (mV)
2	2587	-2242	1.154	511	176	335
3	2718	-2585	1.052	458	118	339
4	2319	-1652	1.404	509	-25	535
5	2189	-1859	1.177	434	-53	487
6	2623	-1627	1.613	454	-195	649
7	1885	-1463	1.288	425	-222	646
8	1938	-1638	1.183	378	-261	639
9	1459	-1399	1.043	279	-212	491
10	1388	-2637	0.526	227	-224	451

The variation of anodic and cathodic peak current and potential as a function of pH is shown in Figure 7 and Figure 8. Each point represents the average of three CV measurements made at each pH. It can be noticed that the current decreases as the pH increases for HGA oxidation. On the other hand, HGA reduction current does not follow any pattern staying approximately stable. For both sweeping directions, oxidation and reduction, standard deviation is high likely because of the low reproducibility of the electrode surface.

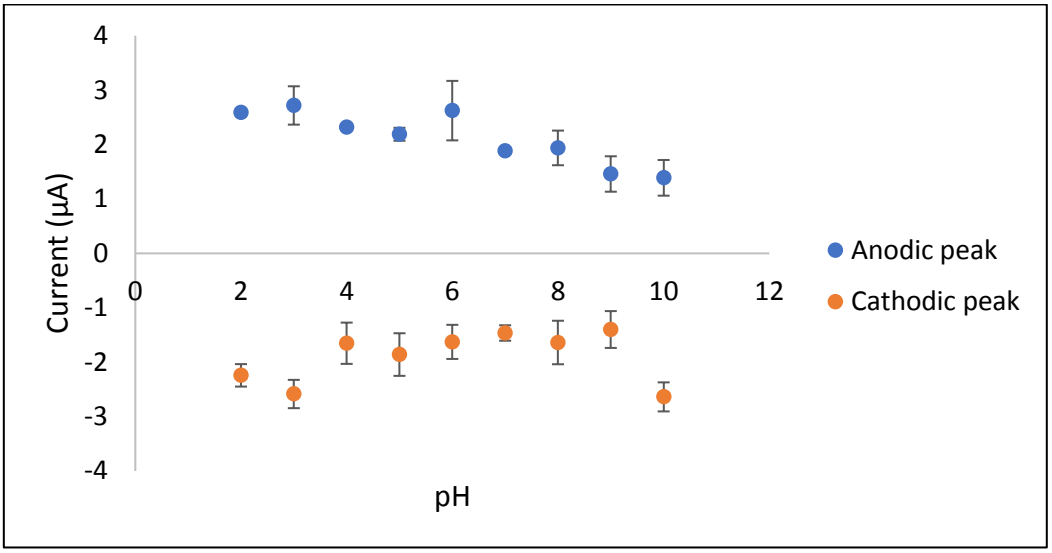


Figure 7. Effect of pH on the current of the anodic and cathodic *CV peaks recorded at 100 mV/s at a carbon paste electrode immersed in 0.05 M of HGA.*

The aspects to highlight from these results are: (1) as the pH increases, the electrochemical window gets narrower on the anodic side; (2) from pH 6 the anodic peak decreases steadily with increasing pH while the cathodic peak does not show the same trend with pH; (3) the I_a/I_c ratio is close to 1 at pH up to 9, but decreases above this pH value because the anodic peak decreases quickly in alkaline media; (4) the diference between the peak potentials of the anodic and cathodic peaks decreases at low pH, thus suggesting that the reversibility of the electrodic reaction improves in acidic solutions.

If the reaction was reversible, the Nernst equation would be satisfied:

$$E = E_0^0 + \frac{0.059}{n}\log\frac{|Ox||H^+|^x}{|Red|} \Rightarrow E = E_0^0 - \frac{0.059\,x}{n}pH + \frac{0.059}{n}\log\frac{|Ox|}{|Red|} = K - \frac{0.059\,x}{n}pH$$

Consequently, when representing peak potential as a function of pH, a straight line should be obtained whose slope informs about the number of protons and electrons exchanged. Figure 8 indicates that the number of protons and electrons exchanged is equal because the slope of E_a *vs. pH* at pH values above 6 is -0.059 V. At lower pH values the slope decreases to 0.018. On the other hand, the cathodic peak in Figure 8 shows a straight line with a slope higher than -0.059 V which points out that the number of protons and electrons exchanged in HGA reduction is not equal and neither constant likely because the reaction is not fully reversible but quasi-reversible.

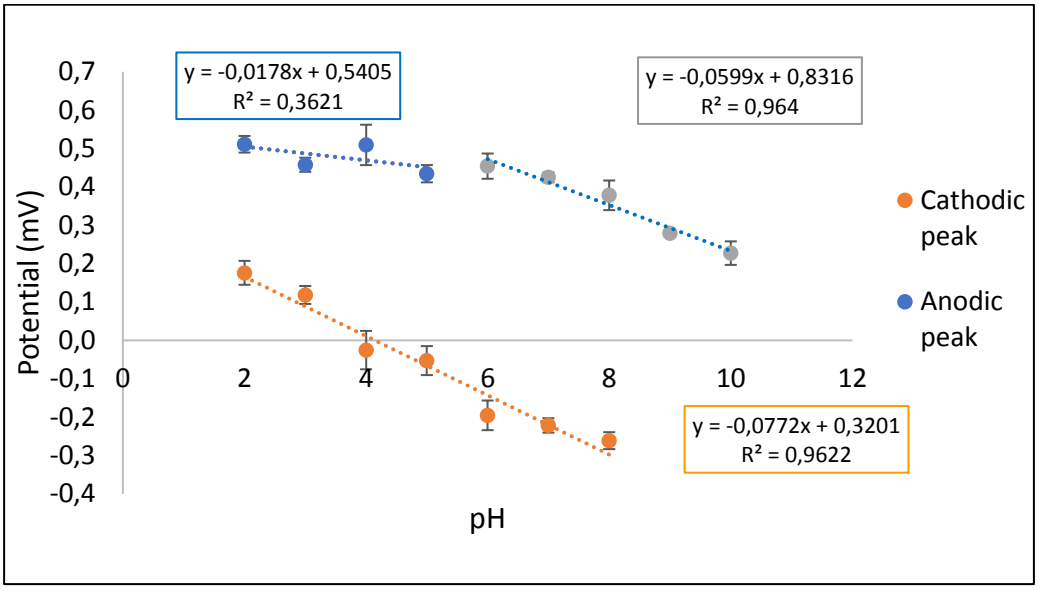
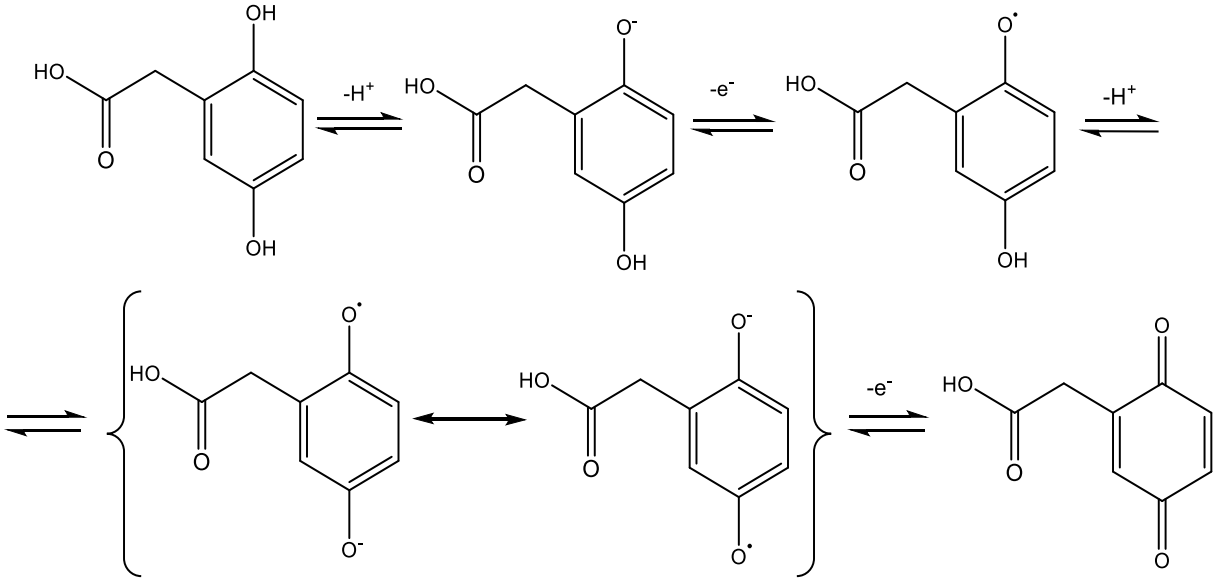


Figure 8. *Effect of pH on the potential of the anodic and cathodic CV peaks recorded at 100 mV/s at a carbon paste electrode immersed in 0.05 M of HGA*

From the above discussion, the following redox mechanism for HGA oxidation in acidic media at the surface of a CPE has been proposed:



Also, from the cyclic voltammograms and from Figure 8 it can be concluded that the amperometric determination of HGA based on the oxidation of the compound at a CPE can be carried out at potentials above 0.5 V.

5.2. HPLC

5.2.1. pH of the mobile phase and potential of the amperometric detector

In order to achieve a good separation of the analyte from other matrix components and an optimal response from the amperometric CPE detector, the effects of the mobile phase pH and of the potential applied to the CPE on the peak area and retention time of HGA were investigated. These optimization experiments were carried out on a standard solution containing $1 \cdot 10^{-4}$ M HGA in Britton-Robinson buffer.

Figure 9 shows chromatograms of HGA obtained at three pH values with amperometric detection at 0.7 V. The retention time decreases with increasing pH (see Table 7) while the peak area remains nearly unaffected (see Figure 10 and discussion).

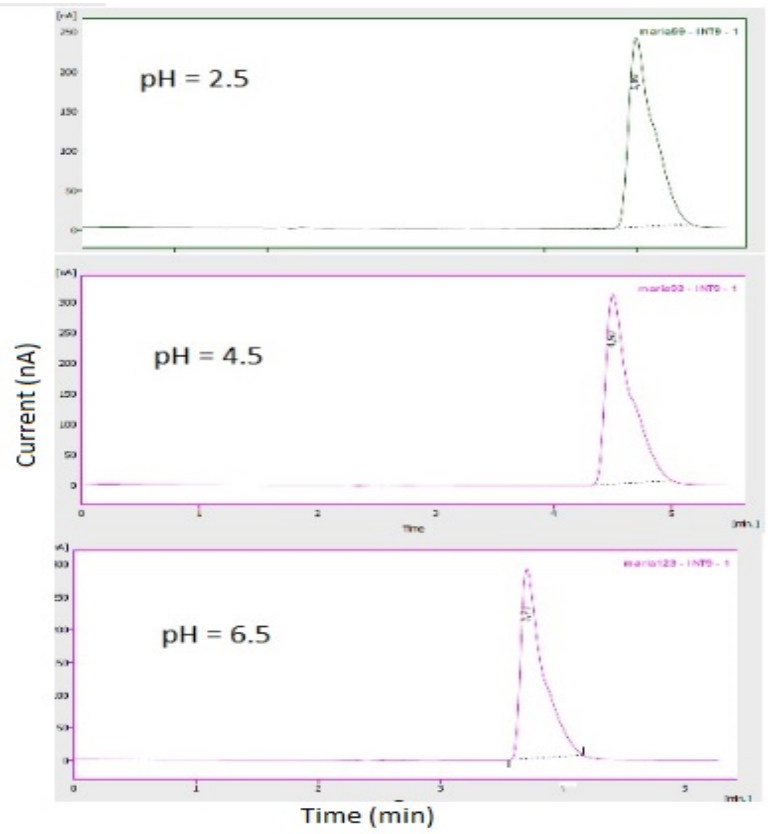


Figure 9. *Effect of mobile phase pH on the HPLC-EC chromatograms obtained at 0.7 V.*

Table 7. *HGA retention time at different pH values in HPLC-EC.*

pH	Retention time (s)
2.5	5.97
4.5	4.54
6.5	3.70

The optimum retention time was selected as a compromise to avoid long measuring times while achieving a good separation from saline components present in urine samples that could elute at short retention times. In consequence, pH 2.5 was selected as optimum pH of the mobile phase.

One of the main parameters to be considered during the method optimization is the potential applied to the amperometric detector at which the oxidation of eluted HGA occurs. The dependence of the electrode response with the potential is shown in Figure 10 (peak area vs. *E*) and Figure 11 (peak height vs. *E*). Both figures show that the oxidation current increases

steeply up to 0.5 V, and from this potential the registered current levels off. The comparison of both figures indicates that peak area dependence with potential is not influenced by the pH, while it exerts a noticeable effect on peak height, which increases with increasing pH showing that peaks get wider as the pH decreases, in correlation with increasing retention time. Therefore, peak area seems to be preferable as response for quantitative analysis of HGA by HPLC-EC.

The background current increases with electrode potential, which is translated to a chromatogram with high noise and baseline instability. On the other hand, the oxidation current decreases and therefore the sensitivity of the method is drastically reduced. Therefore, a potential of 0.6 V was chosen for further measurements.

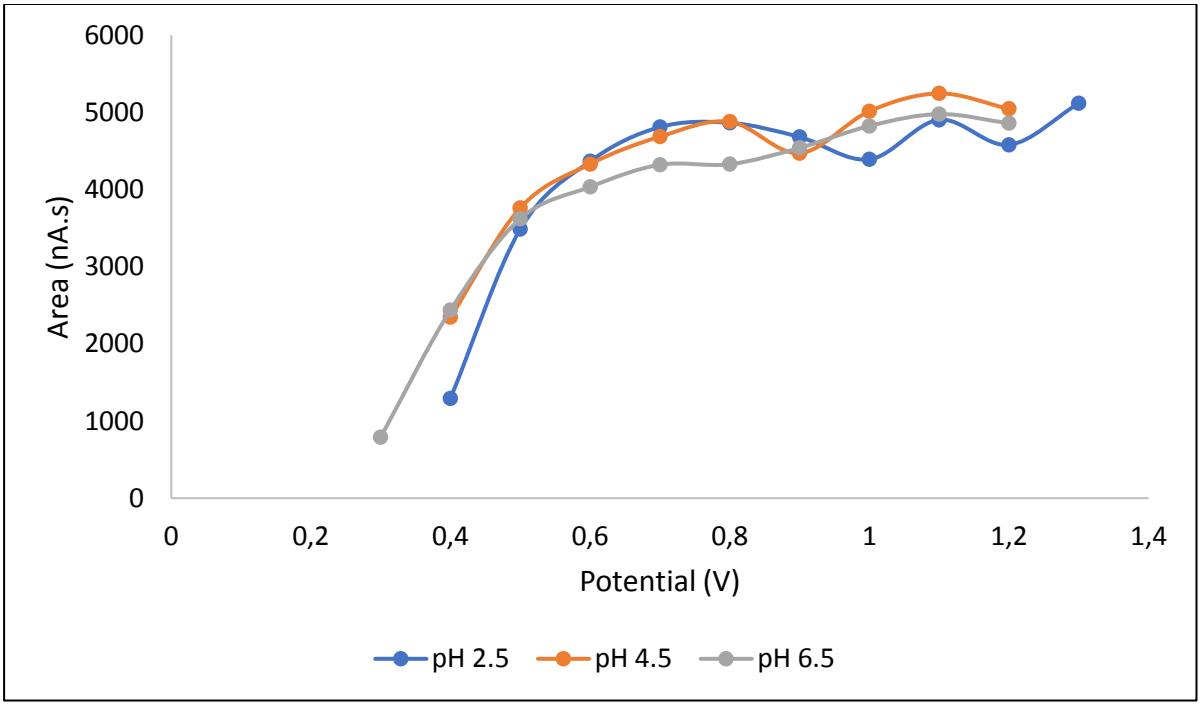


Figure 10. *Peak area vs. potential for the determination of HGA by HPLC with amperometric detector.*

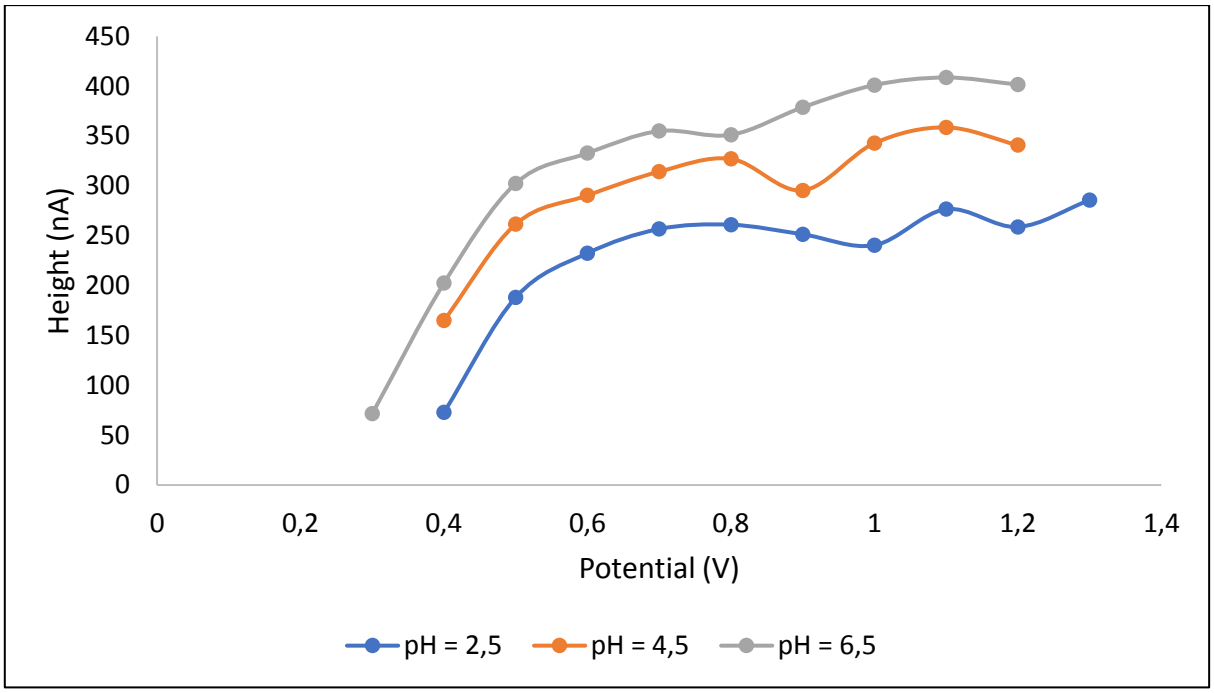


Figure 11. *Peak height vs. potential for the determination of HGA by HPLC with amperometric detector.*

5.2.2. Repeatability

Repeatability of the HPLC-EC measurement was estimated as relative standard deviation of the peak area and peak height registered for 15 repeated injections of $1 \cdot 10^{-4}$ M HGA (16.8 mg/L). The RSD values were compared with those obtained by HPLC with UV detection in the conditions described in the Experimental section. Table 8 shows the obtained values.

Table 8. *RSD (%) of 15 repeated measurements of HGA by HPLC with UV and amperometric detection*

Detection method	Peak area	Peak height
HPLC-EC	4.3	5.9
HPLC-UV	2.8	5.8

Values below 5% are usually considered acceptable for quantification purposes; therefore, area repeatability fulfills this requirement with both UV and EC detectors, while repeatability of peak height measurements is above 5%. As a result, only peak area will be used for calibration and quantification.

5.2.3. Linearity

Standard solutions of HGA in deionized water were prepared covering a concentration range from $6 \cdot 10^{-7}$ M to $1 \cdot 10^{-4}$ M (0.1 to 16.8 mg/L). The standard solutions were measured by HPLC-EC and HPLC-UV in the optimized conditions. The resulting calibration plots are displayed in Figures 12 and 13. Statistical parameters of the regression lines are collected in Table 9. Slope and intercept are accompanied by the respective confidence intervals.

To evaluate the linearity of the regression lines the residuals plots were obtained and are inserted within the respective calibration graph. In both cases the residuals are homocedastic (independent from concentration level) and randomly dispersed around zero, thus indicating that the regression models obtained for HGA by HPLC-EC and HPLC-UV are linear and therefore appropriate for calibration within the concentration range assayed.

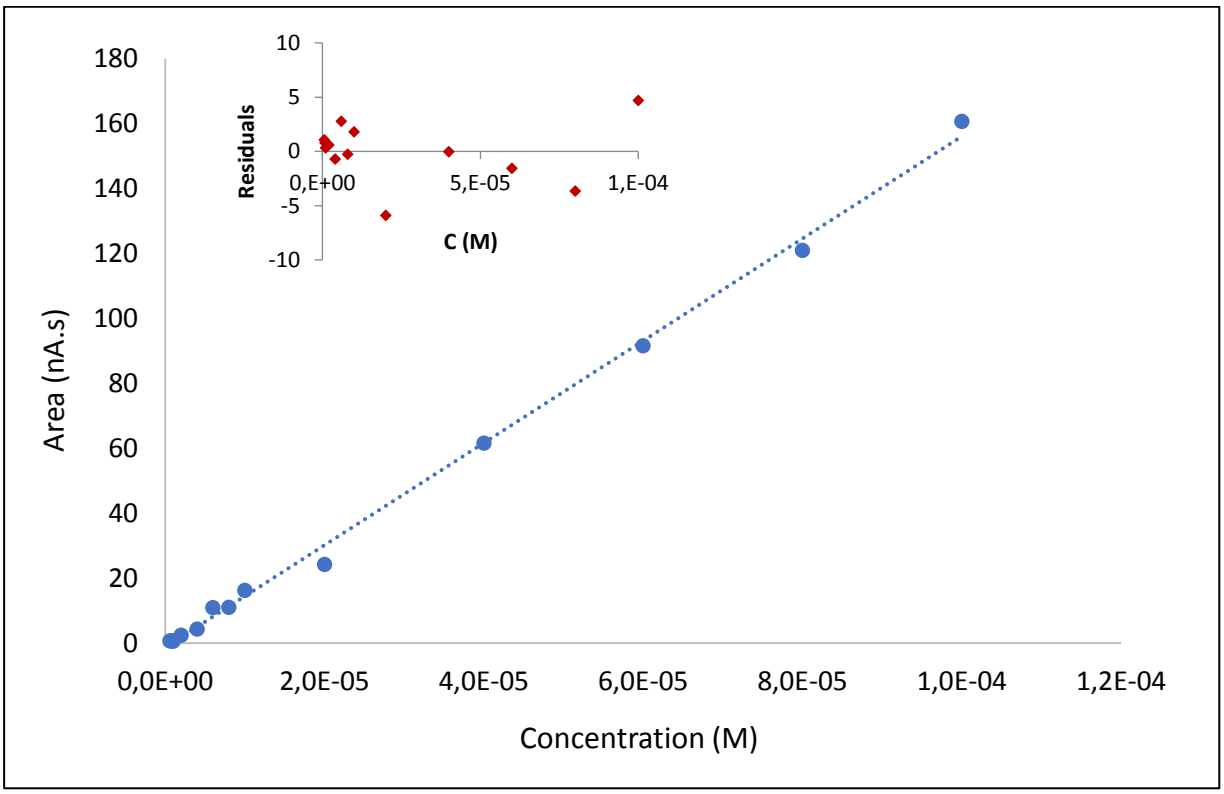


Figure 12. *Calibration plot for HGA by HPLC using UV detector. Insert: residuals plot.*

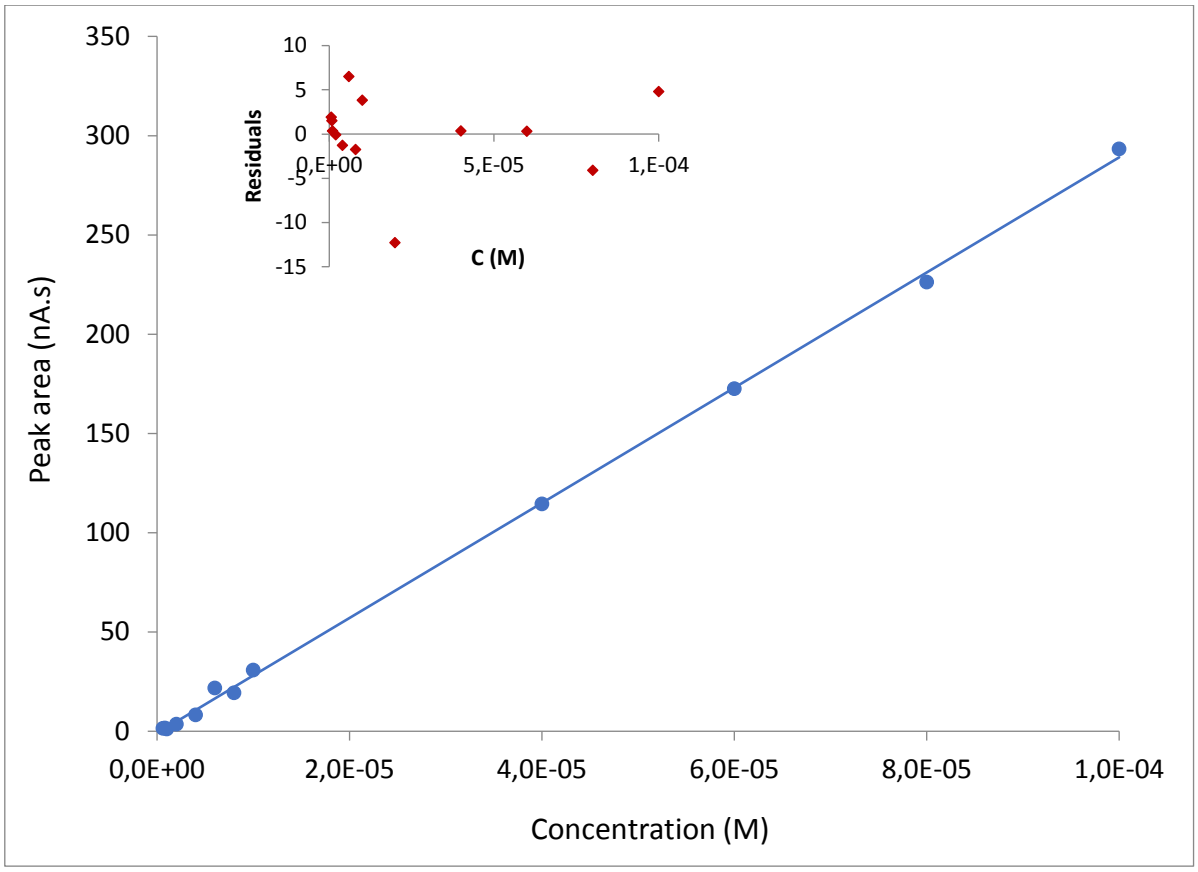


Figure 13. *Calibration plot for HGA by HPLC using amperometric detector. Insert: residuals plot.*

Table 9. *Statistical parameters of the regression lines.*

	Amperometric detector	**UV detector**
Slope	(2.91 ± 0.09) ·10^6	(1.57 ± 0.05) ·10^6
Intercept	-2.15 ± 3.77	-1.28 ± 2.18
Correlation coefficient, R	0.9989	0.9987

5.2.4. Sensitivity

Sensitivity is defined as the capacity to produce a big change of signal with a small change in concentration and is estimated as the slope of the calibration curve. The calibration curve also allows to calculate the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ are the minimum amounts of analyte that can be detected and quantified, respectively, with sufficient precision and accuracy.

LOD and LOQ have been calculated according to the following equations:

$$LOD = \frac{3 \times SD}{m}$$

$$LOQ = \frac{10 \times SD}{m}$$

were SD is the standard deviation of the peak area corresponding to the most diluted HGA standard ($6 \cdot 10^{-7}$ M) and m is the slope of the calibration line.

Table 10 displays the obtained LOD and LOQ for HGA determination by HPLC with the different detectors employed. The method already optimized has a LOD similar to those obtained by other authors in urine samples and standard solutions using chromatographic methods (see Table 2 in the Introduction section). Nevertheless, low LOD and LOQ are not crucial analytical performance parameters in this case since patients suffering from AKU have high HGA concentrations in urine (50 mg/L) [18].

Table 10. *Values of instrumental LOD and LOQ for the employed HPLC detectors.*

	Amperometric	**UV**
SD	1.3	0.064
Slope	$2.9 \cdot 10^{7}$	$1.6 \cdot 10^{6}$
LOD	$1.3 \cdot 10^{-7}$ M (22 µg/L)	$1.2 \cdot 10^{-7}$ M (20 µg/L)
LOQ	$4.4 \cdot 10^{-7}$ M (74 µg/L)	$4.0 \cdot 10^{-7}$ M (67 µg/L)

5.2.5. Urine sample

Isocratic elution does not provide sufficient resolution of the peak if HGA is to be determined in a more complex matrix like urine. Therefore, it was necessary to search for the optimum gradient elution in such a way that the separation has a good resolution and the retention time is not very long. Optimum separation was achieved using the gradient elution conditions shown in Table 4. Gradient elution was not used with pure HGA standards.

To evaluate the matrix effect on the determination of HGA by HPLC-EC, a series of HGA standards were prepared in urine by spiking aliquots of a filtered urine sample from a healthy student with known amounts of HGA. Spiked samples were then measured by HPLC-EC with gradient elution and the peak areas registered. Results were fitted to a straight line by linear regression. The calibration plot obtained in urine matrix is displayed in Figure 14 and the regression parameters in Table 11. As in the case of aqueous solutions, the residuals are random and homocedastic, thus demonstrating the linearity of the calibration graph. The matrix effect on the chromatographic determination of HGA is observable in chromatograms shown in Figure 15 and Figure 16.

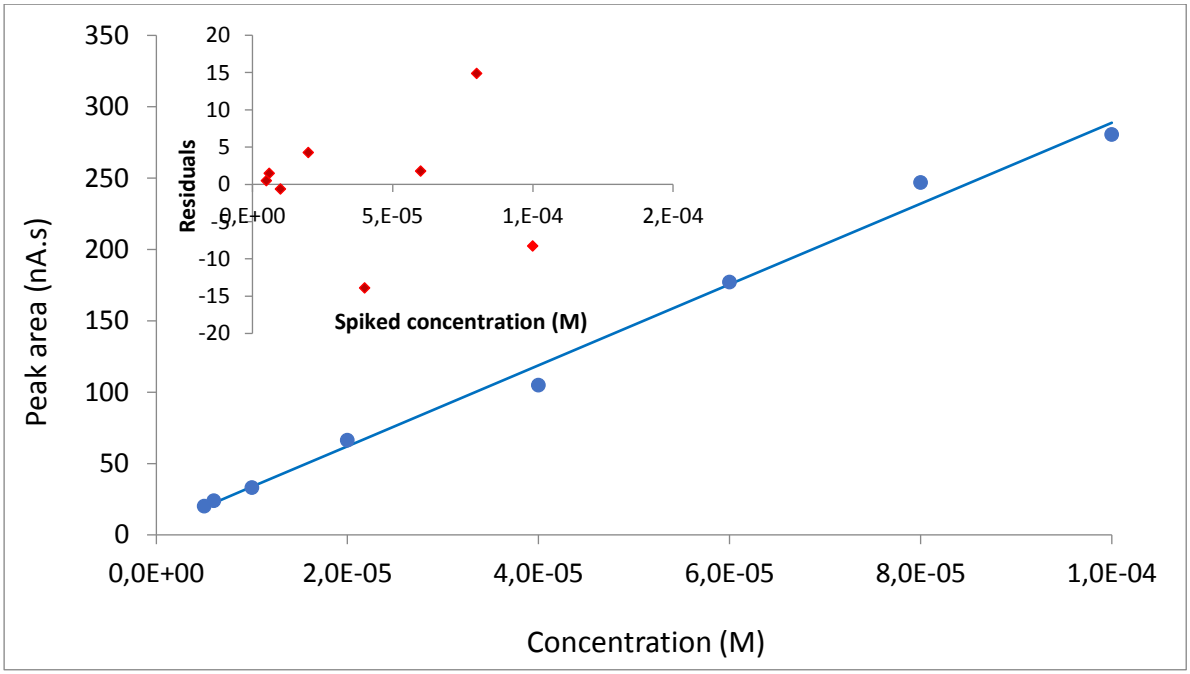


Figure 14. *Chromatographic peak area vs added concentration in urine samples.*

Table 11. *Statistical parameters of the regression line of HGA in urine matrix by HPLC-EC*

Slope	$(2.83 \pm 0.23) \cdot 10^6$
Intercept	5.38 ± 12.28
Correlation coefficient, R	0.9966

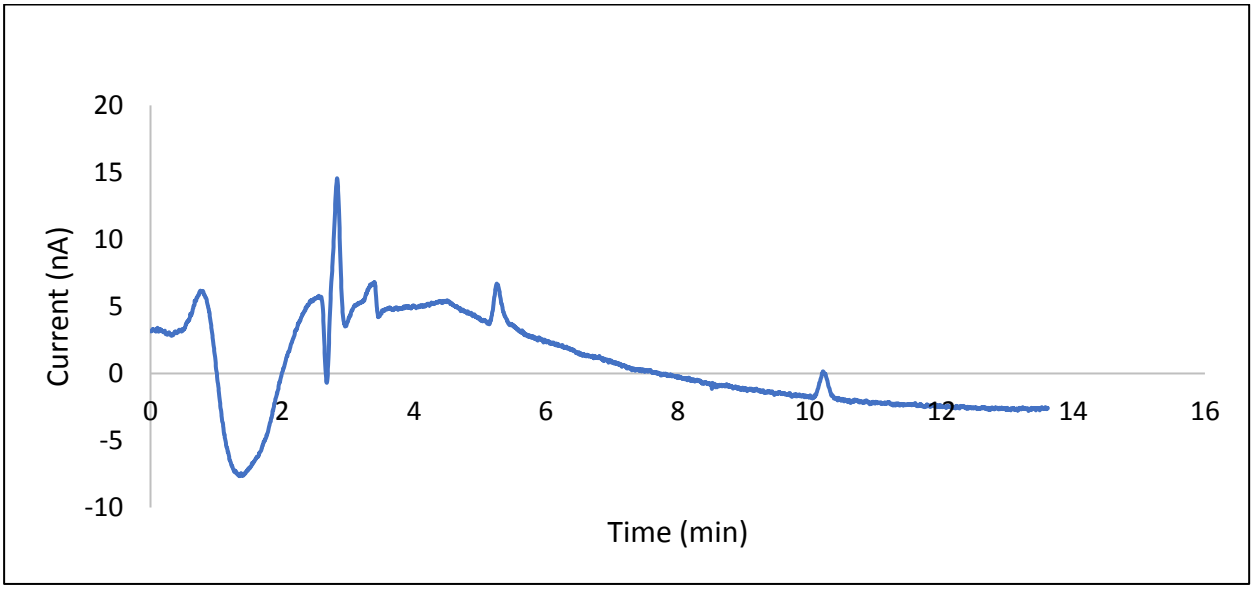


Figure 15. *Chromatogram of a urine sample spiked with $2 \cdot 10^{-6}$ M HGA using HPLC with CPE detector.*

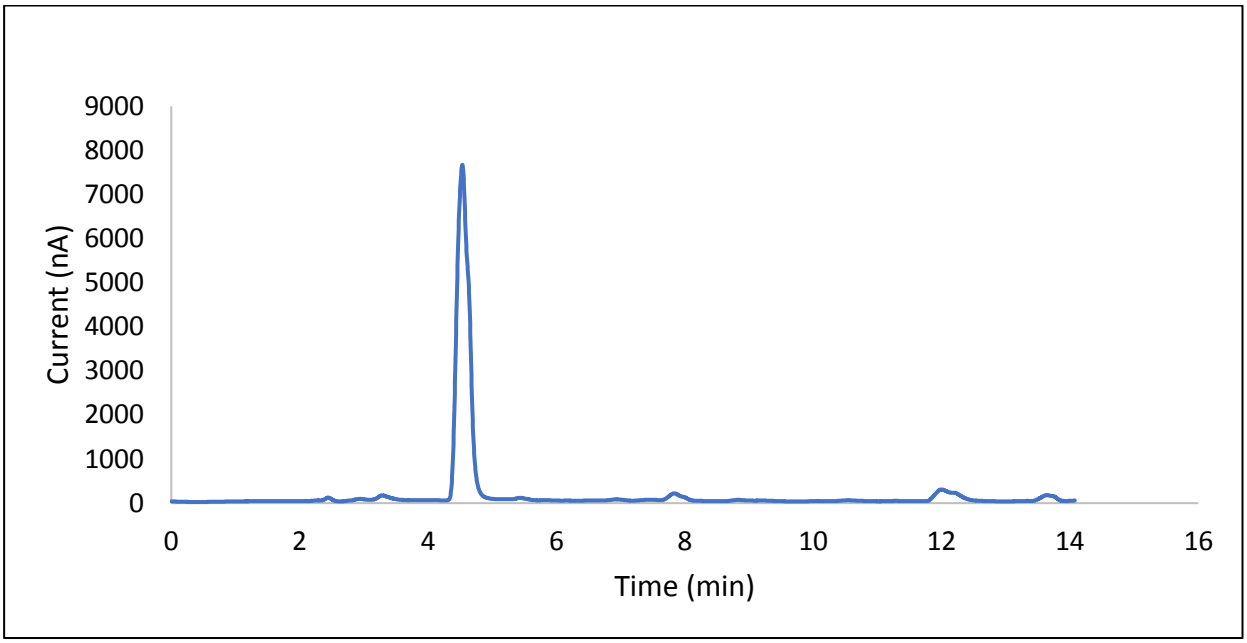


Figure 16. *Chromatogram of a urine sample spiked with $5 \cdot 10^{-6}$ M (8.4 µg/L) HGA using HPLC with CPE detector.*

If a matrix effect is present, the standard additions method should be applied to quantify HGA in real urine samples, with the subsequent increase in analytical cost and time. The effect of urine matrix on the detector response was evaluated by comparing the slopes of the calibration plots obtained in water and urine, which are jointly plotted in Figure 17. Although the slopes look nearly equal in the plot, a statistical t-test was applied to evaluate if the slight difference is significant. The null hypothesis is that slopes are equal and the alternative hypothesis is that slopes are significantly different.

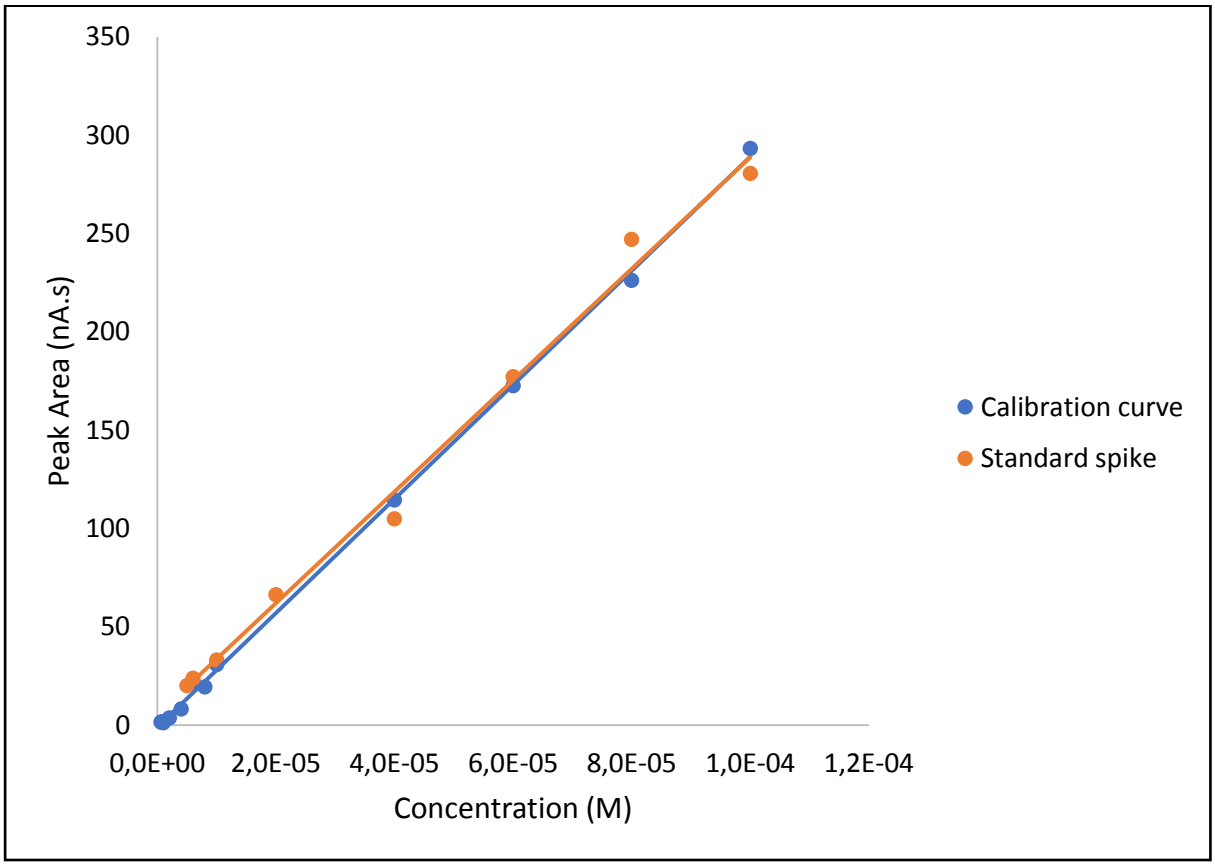


Figure 17. Standard spike curve and calibration curve slope comparison

The residual variances of both regression lines were demonstrated to be not comparable by the Fisher F-test:

$$F = \frac{s_{e1}^2}{s_{e2}^2} = \frac{9.19^2}{3.08^2} = 8.89 > \quad F_{critical} = 3.22$$

As the residual variances are not comparable, the calculated t value is obtained using the following equation:

$$t_{cal} = \frac{|m_1 - m_2|}{\sqrt{s_{m1}^2 + s_{m2}^2}} = 0.67$$

$$t_{crit} = \frac{t_1 s_{m1}^2 + t_2 s_{m2}^2}{s_{m1}^2 + s_{m2}^2} = 2.43$$

where m and s_m symbolize the compared slopes and their standard deviations. Since calculated t is smaller than the critical value the null hypothesis is accepted and it is concluded that there are no significant differences between the slopes of both curves, therefore the matrix effect can be neglected and the quantification of HGA in urine samples can be done by interpolation on the calibration plot obtained with pure HGA standards.

6. CONCLUSIONS

A procedure has been optimized for the detection and quantification of HGA in urine of AKU patients. It has been demonstrated that the HPLC method with carbon paste electrode as the detector allows a rapid screening and quantification of HGA in human urine.

Limits of detection and quantification for the HPLC-EC developed method are 22 µg/L and 74 µg/L, respectively. These limits are sufficiently low to quantify HGA in persons suffering from AKU since the concentration in affected patients is $5 \cdot 10^4$ µg/L. It is also remarkable that HGA determination with HPLC-EC is an alternative method to others requiring more expensive instrumentation and longer sample preparation.

7. TABLES AND FIGURES SUMMARY

7.1. Tables

7.2. Figures

Figure 4. – Anodic peak current variation of 0.05 M HGA in Britton-Robinson buffer at pH 2.5 as a function of the square root of scan rate.

Figure 5. - Logarithmic relationship between anodic peak current and scan rate of 0.05 M HGA at pH 2.5.

Figure 6. - Cyclic voltammograms obtained at 100 mV/s sweep rate for $1 \cdot 10^{-4}$ M HGA in Britton- Robinson buffer recorded at different pH values.

Figure 7. - Effect of pH on the current of the anodic and cathodic CV peaks recorded at 100 mV/s at a carbon paste electrode immersed in 0.05 M of HGA.

Figure 8. - Effect of pH on the potential of the anodic and cathodic CV peaks recorded at 100 mV/s at a carbon paste electrode immersed in 0.05 M of HGA.

Figure 9. - Effect of mobile phase pH on the HPLC-EC chromatograms obtained at 0.7 V.

Figure 10. - Peak area vs. potential for the determination of HGA by HPLC with amperometric detector.

Figure 11. - Peak height vs. potential for the determination of HGA by HPLC with amperometric detector.

Figure 12. - Calibration plot for HGA by HPLC using UV detector. Insert: residuals plot.

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Figure 14. - Chromatographic peak area vs added concentration in urine urine samples.

Figure 15. - Chromatogram of a urine sample spiked with $2 \cdot 10^{-6}$ M HGA using HPLC with CPE detector.

Figure 16. - Chromatogram of a urine sample spiked with $5 \cdot 10^{-6}$ M (8.4 µg/L) HGA using HPLC with CPE detector.

Figure 17. - Standard spike curve and calibration curve slope comparison.

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