



Analytical Methods

Determination of mutagenic amines in water and food samples by high pressure liquid chromatography with amperometric detection using a multiwall carbon nanotubes-glassy carbon electrode



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ARTICLE INFO

Article history:

Received 20 April 2014

Received in revised form 8 December 2014

Accepted 7 July 2015

Available online 8 July 2015

Keywords:

Amperometric detection

Modified electrode

Multiwall carbon nanotubes

Liquid chromatography

Mutagenic amines

ABSTRACT

A chromatographic method, using amperometric detection, for the sensitive determination of six representative mutagenic amines was developed. A glassy carbon electrode (GCE), modified with multiwall carbon nanotubes (GCE-CNTs), was prepared and its response compared to a conventional glassy carbon electrode. The chromatographic method (HPLC–GCE-CNTs) allowed the separation and the determination of heterocyclic aromatic amines (HAAs) classified as mutagenic amines by the International Agency for Research of Cancer. The new electrode was systematically studied in terms of stability, sensitivity, and reproducibility. Statistical analysis of the obtained data demonstrated that the modified electrode provided better sensitivity than the conventional unmodified ones. Detection limits were in the 3.0 and 7.5 ng/mL range, whereas quantification limits ranged between 9.5 and 25.0 ng/mL were obtained. The applicability of the method was demonstrated by the determination of the amines in several types of samples (water and food samples). Recoveries indicate very good agreement between amounts added and those found for all HAAs (recoveries in the 92% and 105% range).

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1. Introduction

Heterocyclic aromatic amines (HAAs) are formed during the heating process of organic products containing nitrogenous compounds, mainly proteins. These compounds contain between two to five (generally three) condensed aromatic cycles with one or more nitrogen atoms in their ring system and, usually, one exocyclic amino group. The exact structure of the studied compounds is shown in Fig. 1, as well as their scientific name and their acronyms. The HAAs are mutagenic for bacteria and some mammalian cell systems and can produce chromosomal aberrations and sister chromatid exchanges in cultured cells. In 1993, the International Agency for Research on Cancer (IARC) (IARC, 1993) considers four of the studied HAAs (A α C, MeA α C, Trp-P-1 and Trp-P-2) as possible human carcinogens and recommends a reduce exposure to these compounds. These HAAs have been isolated from proteinaceous foods including cooked meats and fish, meat extracts or process flavours. They are also present in cooking fumes (Yen & Hsieh, 1994), several foods (Knize, Kunningham, Griffin, Jones, & Felton, 1994) coffee (Casal, Mendes, Fernandes,

Oliveira, & Ferreira, 2004), alcohol beverages (Richling, Decker, Haring, Herderich, & Schraier, 1997), and from environmental sources, such as cigarette smoke (Manabe et al., 1993), air (Manabe et al., 1993), river and rain water (Wu, Wong, Lee, & Ong, 1995). Also, some HAAs have been detected in human tissues (Prabhu et al., 2001), hair (Hegstag et al., 2000), and in biological fluids, such as plasma, urine or bile (Friesen, Garren, Bereziat, Kadlubar, & Lin, 1993), as well as in milk of healthy women (DeBruin, Martos, & Josephy, 2001).

Owing to concern over HAAs, a number of analytical methods have been proposed to separate and detect these compounds in different samples. The most commonly used methods include GC–MS after derivatization step (Kataoka & Kijima, 1997), HPLC using UV (Gross & Gruter, 1992; Janoszka et al., 2001), fluorescence (Martín-Calero, Ayala, Gonzalez, & Afonso, 2007; Ristic, Cichna, & Sontag, 2004), electrochemical (ED) (Bermudo, Ruiz Calero, Puignou, & Galceran, 2005; Martín-Calero, Pino, Ayala, Gonzalez, & Afonso, 2009), or mass spectrometric (Guy, Gremaud, Richoz, & Turesky, 2000; Pais, Moyano, Puignou, & Galceran, 1997) detectors. Capillary electrophoresis (CE) with UV (Puignou, Casal, Santos, & Galcerán, 1997), DAD (Fei, Li, Yu, & Chen, 2007; Mardones, Arce, Ríos, & Valcarcel, 1998) or ED detection (Olsson, Dyremark, & Karlberg, 1997) too, has been proposed but high detection limits have been obtained.

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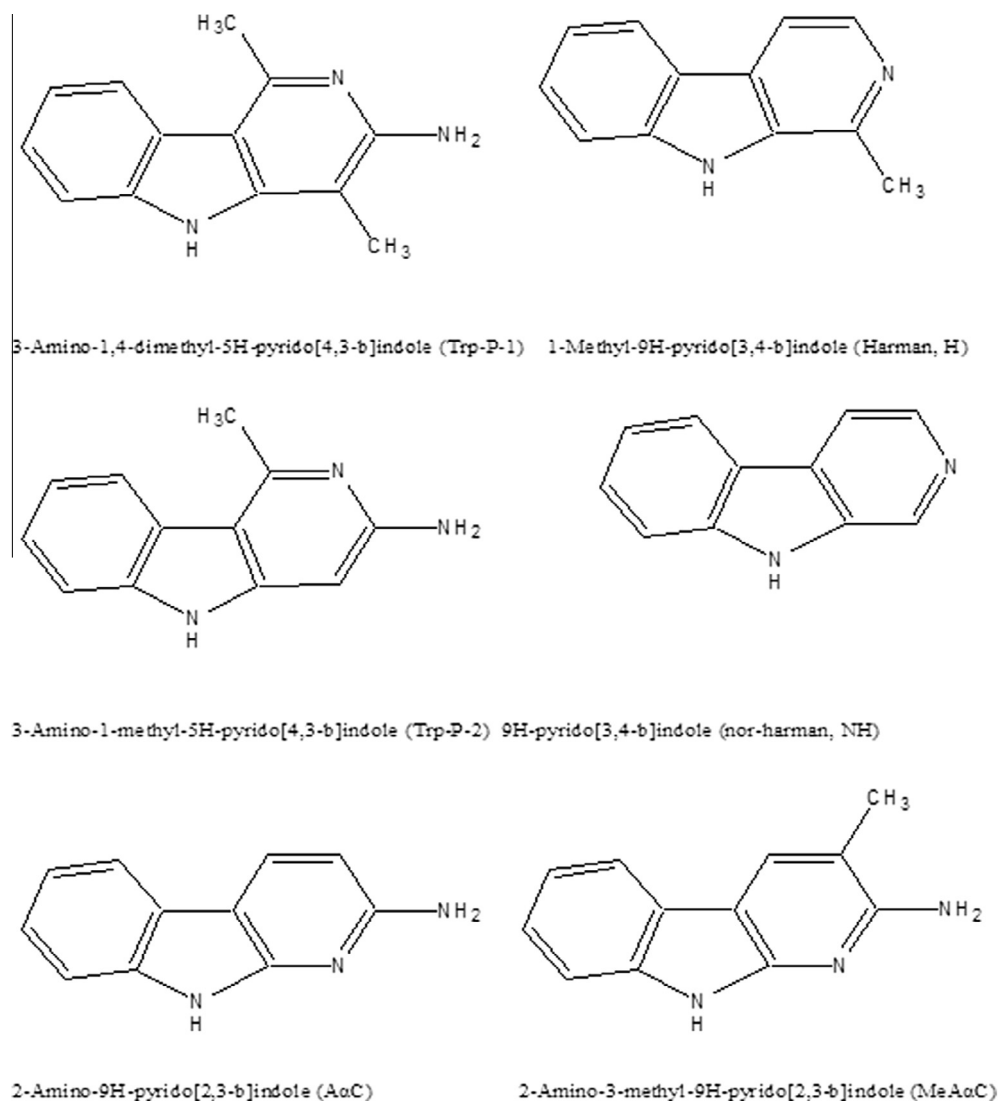


Fig. 1. Chemical structures of the HAAs determined by the proposed method.

Electrochemical detection offers increased sensitivity compared with UV detectors and the selectivity results from the fact that HAAs are oxidized at lower potentials than other compounds (Kataoka, 1997). Most of the impurities detected as overlapping peaks with UV detection are not oxidized at the working potential and do not perturb the detection. This detection mode can be improved by using nanomaterials, because of their unique properties. In fact, recently, many research works have revealed that modification of electrode surface with nanomaterials is a promising avenue. Carbon nanotubes (CNTs), consisting of cylindrical graphene sheets with nanometer diameter, have attracted much attention due to their unique mechanical, chemical and electronic properties. The performance of multiwall carbon nanotubes (MWCNTs) modified electrodes has been found to be superior to the performance of other conventional carbon electrodes in terms of electron transfer, reversibility and conductivity (Baughman, Zakhidov, & de Heer, 2002). In fact, these nanomaterials have been used to modify electrodes (usually glassy carbon electrodes) that have been then applied in the determination of several analytes, such as hydroquinone (Qi & Zhang, 2005), insulin (Zhang, Mullens, & Gorski, 2005), metals (Tsai, Chen, & Marken, 2005; Yuan, He, Yao, & Hu, 2006), isoflavones (Gonzalez Crevillen, Avila, Pumera, Gonzalez, & Escarpa, 2007; Gonzalez Crevillen, Pumera, Gonzalez, & Escarpa, 2009) and phenols (Chicharro, Arribas,

Moreno, Bermejo, & Zapardiel, 2007) in several types of samples. The same authors have recently proposed a screening method for the control of sulfonamides residues in milk samples based on electrochemical detection using MWCNTs-glassy carbon electrode and the obtained results are very satisfactory (Bueno, Contento, & Ríos, 2013).

This work reported, for the first time, the development of a method for the determination of six HAAs based on the amperometric monitoring of their oxidation responses at MWCNT-modified glassy carbon electrode coupled to HPLC system. This new procedure provides increased sensibility in electrochemical detection, improving detection limits previously published with conventional electrochemical techniques. The proposed method has successfully been applied to the analysis of water and foods samples containing low concentrations levels of these compounds previous to a preconcentration step.

2. Experimental

2.1. Apparatus and electrodes

Liquid chromatographic experiments were carried out with a HP 1090 Liquid Chromatograph (Agilent, USA) and amperometric detector (Metrohm 791 VA, GOMENSORO S.A., Spain) using

Labview software. The wall-jet flow-cell consisted of an Ag/AgCl/3 M KCl reference electrode (Metrohm Model 60727000, GOMENSORO S.A., Spain), a platinum auxiliary electrode and GCE (Metrohm Model 60805010, GOMENSORO S.A., Spain, shaft diameter bottom 7 mm) or GCE-CNTs as working electrode.

A Zorbax SB-C18 column (Agilent, USA, 150 × 4.6 mm I.D.; particle size, 3.5 µm) was used for the separation of the compounds. Solution of 0.05 M CH₃COONH₄ pH = 7:ACN(75:25) at room temperature (20 ± 1 °C) was used as the mobile phase in the liquid chromatographic experiments. The potential detection applied was 1000 mV for all measurements. The length of the tubing connecting the HPLC and the detector was 10 cm.

Flow injection system was arranged with a peristaltic pump (Model Minipuls 3, GILSON, France), and a Rheodyne six-ways injection valve with a 20 mL loop. The wall-jet flow-cell and the amperometric detector used as the same described above.

Samples were extracted using an SPE-Vacuum manifold from Supelco (Madrid, Spain). An ultrasonic bath (Ultrasons J.P. Selecta, Barcelona, Spain) was used to clean the surface of the electrodes and homogenization to the solutions.

2.2. Materials and standards

1-metil-9H-pirido[3,4-b]indole (H), 9H-pirido[3,4-b]indole (NH), 2-amino-9H-pirido[2,3-b]indole (AαC), acetate 3-amin o-1,4-dimetil-5H-pirido[4,3-b]indole (Trp-P-1), 3-amino-1-metil-5H-pirido[4,3-b]indole (Trp-P-2), 2-amino-3-metil-9H-pirido[2,3-b]indole (MeAαC) were purchase from Toronto Research Chemicals Inc. (North York, ON Canada). Acid trichloroacetic and ammonium acetate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Multi-walled carbon nanotubes with 95% purity were obtained from NanoLab (Brighton, MA). Methanol and acetonitrile were of HPLC grade and acquired from Panreac (Barcelona, Spain). Nafion was supplied from Fluka (USA). In all cases water was of high quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Stock standard solution (1 mg mL⁻¹) of each amine was prepared in MeOH and stored at -20 °C. Working standard solutions were prepared daily by diluting the stock solutions with the mixture used as mobile phase (0.05 M CH₃COONH₄ pH = 7:ACN (75:25)).

2.3. Preparation of the CNTs dispersion and GCE-CNTs

The carbon nanotubes solution was obtained by dispersing 1.0 mg of MWCNTs in 1.0 mL of 0.1% (v/v) Nafion solution followed by sonication for 15 min. The GCE surface was polished with alumina, rinsed with distilled water, sonicated into water and acetone with an ultrasonic bath and dried in air. An aliquot (10 µL) of the dispersion was dropped on the electrode surface and the solvent was evaporated under an infrared heat lamp (Vega, Agüi, Gonzalez-Cortes, Yanez-Sedeño, & Pingarron, 2007).

2.4. Preparation of samples

2.4.1. Water Samples

Tap water (Ciudad Real) and river water (Segovia) were used for the analysis. These samples were collected using glass bottles pre-rinsed with ultra-pure-water and stored at 5 °C. 10 mL of each sample was doped with the corresponding amount of studied HAAs and applied to a Strata-X cartridge pre-conditioned with 5 mL of methanol and 10 mL of water. The cartridge was washed with 5 mL of methanol:water (5:95) and eluted with 3 mL of methanol:ACN (50:50). The extract was evaporated to dryness under a stream of nitrogen, redissolved with 200 µL of mobile phase and injected into the HPLC–GCE-CNTs system.

2.4.2. Food samples

Aneto broth (Hacendado, Mercadona) was acquired in a local market. This sample was deproteinized with 20% (p/v) trichloroacetic acid. The pretreatment consisted of adding 5 mL of this acid solution and the accurately measure of amounts of each standard amine solution to 10 mL of sample and waiting 15 min until a precipitate was formed. Then, the sample was filtered with Millipore filters and applied to a Strata-X cartridge pre-conditioned with 5 mL of methanol and 10 mL of water. The cartridge was washed with 5 mL of methanol:water (15:85) and eluted with 3 mL of methanol:ACN (50:50). The extract was evaporated to dryness under a stream of nitrogen, reconstituted with 200 µL of mobile phase and injected into the HPLC–GCE-CNTs system.

Commercial beef broth tablets (Hacendado, Mercadona) were acquired in a local market. A tablet (10 g) was dissolved in 500 mL of boiling milli-Q water. A 10 mL of this solution was doped with the studied HAAs and treated by the same procedure explained above for Aneto broth.

Beef meat, was acquired in a local market. 1 g of meat was pulverized and homogenized in 10 mL of NaOH 1 M with sonication, and the suspension was shaken for 1 h using a rotating shaker. Then, the supernatant (alkaline solution) was deproteinized with acid trichloroacetic 20% (p/v) by the same method described for broth samples. 5 mL of the acid trichloroacetic 20% (p/v) and the corresponding amount of the amine was added to 10 mL of sample and waiting 15 min until a precipitate was formed. Then, the sample was filtered with Millipore filters and adjusted to pH 7 with HCl 0.5 M. The sample was filtered again and applied to a Strata-X cartridge pre-conditioned with 5 mL of methanol and 10 mL of water. The cartridge was washed with 5 mL of methanol:water (15:85) and eluted with 3 mL of methanol:ACN (50:50). The extract was evaporated to dryness under a stream of nitrogen, re-solved with 200 µL of mobile phase and injected into the HPLC–GCE-CNTs system.

3. Results and discussion

Due to presence of oxidizable groups of HAAs (Fig. 1), amperometric detection at glassy carbon electrode (GCE) has been used in order to determine these compounds in several samples. Considering the attractive properties of MWCNTs, due to intense catalytic activity towards the electrochemical oxidation, a GCE modified with multiwall carbon nanotubes (GCE-CNTs) as amperometric detector for HPLC has been examined to determine HAAs in several samples.

3.1. Fabrication of the modified GCE-CNTs

To obtain the best analytical response with GCE-CNTs, several parameters related to the preparation process of this modified electrode were studied. All optimization process was carried out by measuring the current value at a potential of 1000 mV after injection of 20 µL aliquots of 3 µg/mL of studied HAAs solutions into the carrier solution consisting of 0.05 M ammonium acetate pH = 7:ACN (75:25) and flow rate of 1.8 mL/min, using a flow system.

Nafion solution was used to disperse the CNTs, and concentrations between 0.5 and 5 mg mL⁻¹ of dispersion of CNTs were tested. Aliquots of 10 µL of the prepared dispersions were properly deposited onto the GCE, and the amperometric response of 3 µg/mL of each HAA was measured after 15 min. Larger concentrations of CNTs dispersion considerably increased the baseline noise, and the maximal current was obtained when dispersions of 1 mg mL⁻¹ was used. Therefore, a dispersion of 1 mg mL⁻¹ of CNTs in Nafion was used to prepare the modified electrode.

Other important parameter in the preparation of the electrode is the amount of the optimized dispersion onto the electrode surface. Thus, different volumes between 5 and 20 μL of 1 mg mL^{-1} CNTs dispersion were cast onto the GCE, and the current response of 3 $\mu\text{g/mL}$ of each HAA was checked. Peak current increased with the volume of CNTs dispersion deposited up to 10 μL , following by a decrease in current signal for larger volumes. As a result, 10 μL of CNTs dispersion was selected for the preparation of the modified GCE. To assess the correct adsorption of CNTs dispersion over the surface electrode, two different methods like to dry in air and to evaporate under an infrared lamp (in order to evaporate the solvent) were tested. The modified electrode was exposed under an infrared lamp between 1 and 20 min time and dried in air. Each condition was measured and compared in relation to the current response obtained for solution of 3 $\mu\text{g/mL}$ of each HAA. Better results were obtained when the solvent was evaporated under an infrared lamp in terms of current signal, but the time of exposition did not show significant differences. Therefore, 10 min was selected because this was the time needed to completely evaporate the solvent from the CNTs dispersion. These optimized conditions were used to prepare the GCE-CNTs in all cases, being necessary to generate a new modified electrode every working day.

3.2. Selection of the amperometric conditions

The choice of the potential to be applied GCE-CNTs for its use as an amperometric detector was established by plotting the S/N ratios from current values measured at different applied potentials, after injection of 20 μL aliquots of 3 $\mu\text{g/mL}$ of studied HAAs solutions into the carrier solution consisting of 0.05 M ammonium acetate pH = 7:ACN (75:25) and flow rate of 1.8 mL/min, using flow system. In this sense, the hydrodynamic behavior of studied amines was carried out and the Fig. 2 shows a S/N -E hydrodynamic voltammetric curve obtained. Each value represents the average of four injections. As it can be seen, the maximum S/N ratio for all the studied compounds was 1.0 V. Therefore, this value of potential was selected for the determination of the studied HAAs. Under these experimental conditions, no cleaning or pre-treatment of the electrode after each injection was required. It was enough to condition the GCE-CNTs at the beginning of the experiments, in order to obtain a fresh electrode surface and no appreciable fouling signals were observed after successive scans.

The modified electrode was regenerated after 20 consecutive injections.

3.3. Optimization of chromatographic conditions

The chromatographic separation of studied HAAs was achieved using HPLC coupled to the optimized GCE-CNTs before as amperometric detector. In preliminary studies various types of C18 columns with different lengths and different diameters of particle were used. The best separation of the six studied HAAs was obtained when a diameter of particle of 3.5 μm and 15 cm of lengths of column were used. Moreover, different mobile binary phases, formed by different ratios of acetonitrile or methanol and buffer solution at several pH values (between 5 and 8) were tested. Only, isocratic conditions of mobile phase are used due to instability of the baseline in electrochemical detection. As a compromise between adequate retention times and good sensitivity when peak areas are measured, acetonitrile:0.05 M ammonium acetate (pH = 7) (25:75) was selected as mobile phase.

The effect of the mobile-phase flow rate was tested between 0.5 and 1.5 mL/min. As expected, both retention time and peak width decreased as the flow rate increased for all the studied compounds. However, a lower resolution between peaks was observed for higher flow rates. Best resolution between all the peaks was obtained when 1.3 mL/min was used. Therefore, this value was selected as optimum. Under these conditions, the retention times ($\text{min} \pm \text{SD}$, $n = 3$) were: 7.25 ± 0.03 (Trp-P-2), 9.98 ± 0.03 (Trp-P-1), 13.87 ± 0.06 (A α C), 16.58 ± 0.08 (H), 18.02 ± 0.11 (NH) and 28.46 ± 0.17 (MeA α C).

3.4. Analytical characteristics of the proposed method. Comparison between unmodified and modified GCE with carbon nanotubes

As preliminary studies, using the optimum chromatographic and amperometric conditions, multiwall carbon nanotubes electrode (GCE-CNTs) and GCE were compared as electrochemical detectors in the liquid chromatography system for the determination of studied amines. External calibration method for the analytes with both electrodes was achieved. Chromatograms were obtained for a series of amine solutions at different concentrations, ranged between 0.05 and 10 $\mu\text{g/mL}$ according to the HAAs involved. The calibration graph of the peak current versus concentration was constructed using data from these measurements and the least squares were evaluated using the linear regression method. A very good linear dependence with the concentration and good values for the coefficient of determination were noticed for all analytes studied with both electrodes. The repeatability for five measurements of the current peak for solutions of 0.5 $\mu\text{g/mL}$ each amine compound, under optimized conditions, was satisfactory, with relative standard deviations lower than 5%. The reproducibility of the current peak was tested over two days using solutions prepared at a concentration of 0.5 $\mu\text{g/mL}$ of each compound, obtaining relative standard deviation values lower than 7%. The detection limit (LOD) and quantitative limit (LOQ) for the studied HAAs under these experimental conditions were obtained from $\text{LOD} = 3S_b/b$ and $\text{LOQ} = 10S_b/b$, when S_b was the standard deviation of the mean value for eight signals of the blank and b was the slope of the calibration graph. All the analytical parameters obtained are summarized in Table 1 using (A) GCE and (B) GCE-CNTs as electrochemical detectors respectively. As it can be seen in this table, the main different between the two electrodes was the sensitivity, in terms of calibration slope, and detection limits, which was clearly better for the modified GCE-CNTs in all cases. Also, these obtained results were compared with those published in previous works (see Introduction), and regarding LOD and

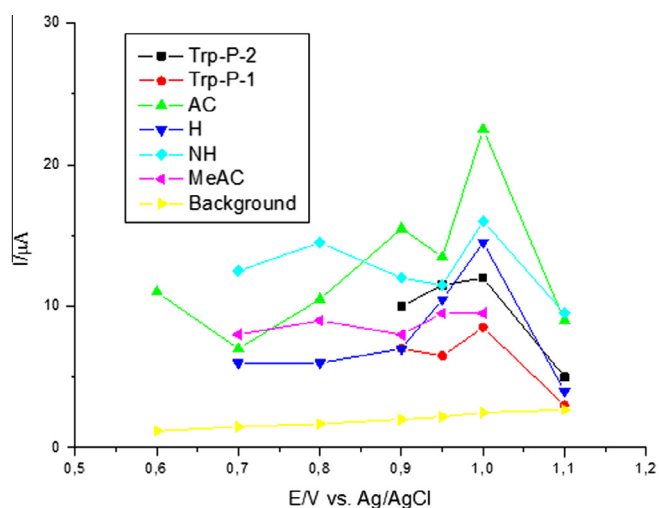


Fig. 2. Hydrodynamic amperometric results for a mixture of 3 $\mu\text{g/mL}$ of HAAs. The average peak current was obtained from injections ($n = 4$) in 0.05 M ammonium acetate pH = 7:ACN (75:25) solution at GCE-CNTs.

Table 1

Analytical parameters using external calibration protocol.

HAA	Lineal range (μg/mL)	R ²	$y = (a \pm ts_a) + (b \pm ts_b)x$	RSD (%) Intra day	RSD (%) Inter day	LOD (μg/mL) ¹	LOQ (μg/mL) ²
<i>(A) By using the GCE</i>							
Trp-P-2	0.05–4.7	0.994	$y = (-4.1 \pm 0.7) + (2.3 \pm 0.2)x$	4.8	6.5	0.05	0.15
Trp-P-1	0.07–8.9	0.994	$y = (4.8 \pm 0.7) + (1.9 \pm 0.1)x$	5.2	6.8	0.03	0.09
AαC	0.06–10.2	0.996	$y = (5.2 \pm 1.8) + (2.9 \pm 0.3)x$	5.3	6.7	0.02	0.06
H	0.04–9.5	0.992	$y = (0.2 \pm 1.5) + (1.9 \pm 0.2)x$	5.6	7.2	0.03	0.09
NH	0.05–10.1	0.998	$y = (1.3 \pm 0.5) + (2.0 \pm 0.1)x$	4.9	6.7	0.02	0.08
MeAαC	0.05–8.3	0.994	$y = (-0.9 \pm 1.0) + (2.0 \pm 0.2)x$	5.5	7.1	0.03	0.09
<i>(B) By using the GCE-CNTs</i>							
Trp-P-2	0.05–9.3	0.992	$y = (-1.7 \pm 1.0) + (5.7 \pm 0.2)x$	3.4	5.2	0.003	0.010
Trp-P-1	0.06–9.0	0.994	$y = (-4.9 \pm 1.7) + (3.1 \pm 0.3)x$	4.0	5.9	0.006	0.018
AαC	0.05–10.6	0.994	$y = (-2.8 \pm 1.7) + (4.2 \pm 0.3)x$	4.2	6.1	0.004	0.013
H	0.07–11.1	0.996	$y = (2.3 \pm 0.5) + (2.2 \pm 0.1)x$	3.8	5.5	0.008	0.025
NH	0.03–8.8	0.998	$y = (-7.5 \pm 3.4) + (4.7 \pm 0.7)x$	4.3	5.8	0.004	0.012
MeAαC	0.06–10.5	0.994	$y = (0.3 \pm 1.6) + (2.5 \pm 0.3)x$	4.0	6.0	0.007	0.022

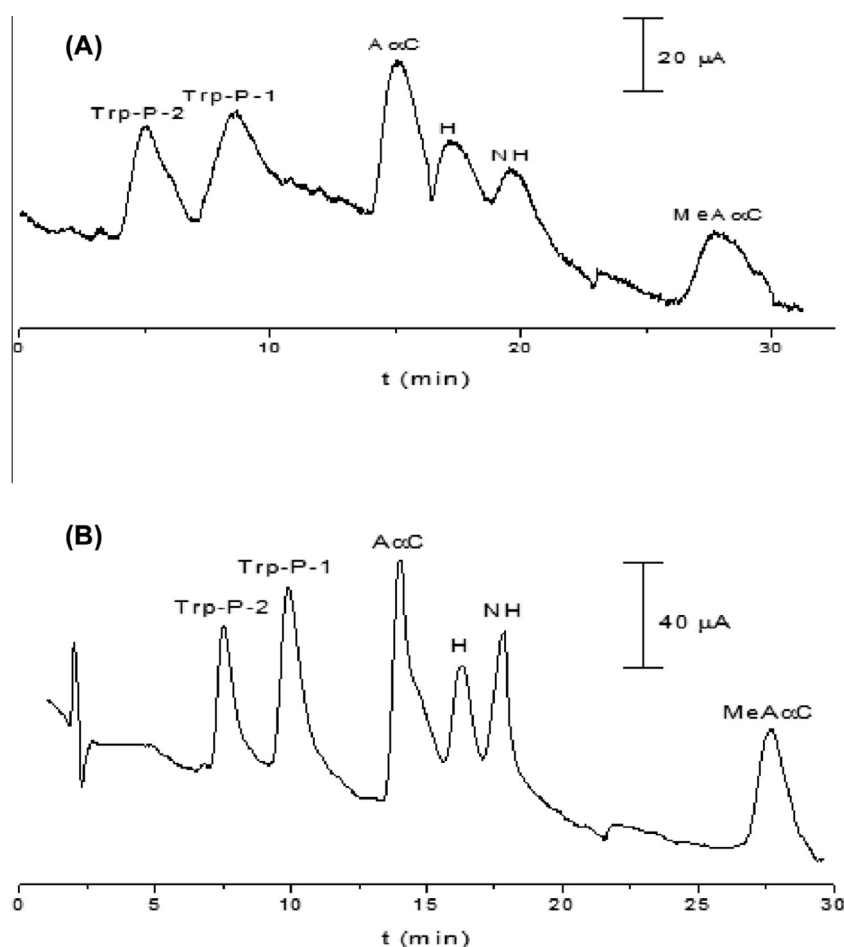
¹ Limit of determination (LOD) expressed as 3s_a/b.² Limit of quantification (LOQ) expressed as 10s_a/b.

Fig. 3. Chromatograms obtained for a standard mixture containing 0.5 μg/mL of each compounds: (A) by using the GCE; and (B) by using the GCE-CNTs. Reference electrode: Ag/AgCl in all cases. Experimental conditions: mobile phase: 0.05 M ammonium acetate pH = 7:ACN (75:25); injection volume: 20 μL; potential detection: 1 V; and flow rate: 1.3 mL/min.

LOQ, only methodology that using MS as detection system slightly exceed the results obtained by this method.

The stabilization of the background current using both electrodes was compared working with the HPLC system. The results demonstrated that while the GCE required a total setting time greater than 30 min, the GCE-CNTs only needed 15 min to obtain a total stabilization of the background current, so it can be concluded that modified electrode exhibit some resistance to oxidative

attack. The long stabilization time for GCE are believed to be due to oxidation of the electrode itself, together with oxygen evolution (Preechaworapun et al., 2006).

Fig. 3 shows the chromatograms obtained for GCE and GCE-CNTs vs. Ag/AgCl of a standard mixture containing 0.5 μg/mL concentrations for each compound. It can be seen the improvement achieved in sensitivity and peak shape when the modified electrode was used with respect to the conventional one.

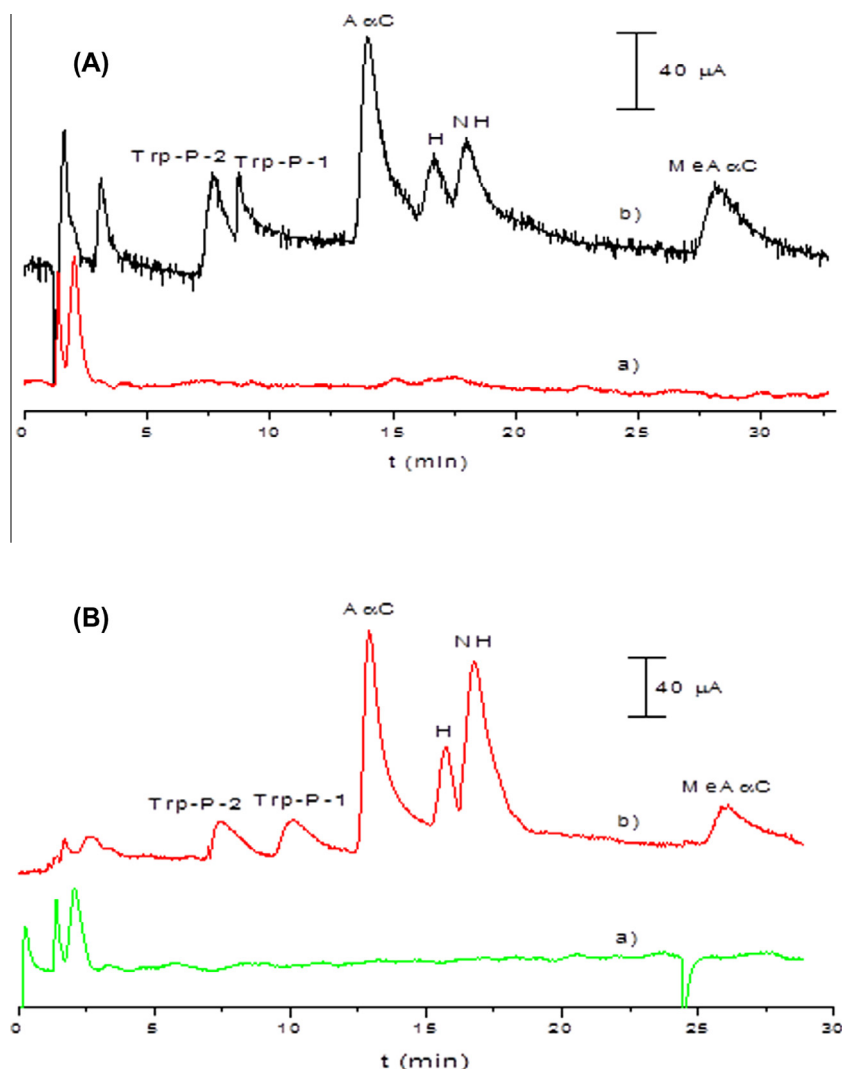


Fig. 4. Chromatogram for beef meat sample obtained with GCE-CNTs vs. Ag/AgCl. (a) Sample without spiked compounds. (b) Sample doped with HAAs. Experimental conditions: mobile phase: 0.05 M ammonium acetate pH = 7:ACN (75:25); injection volume: 20 μL; potential detection: 1 V; and flow rate: 1.3 mL/min.

3.5. Clean-up procedure of samples

In order to demonstrate the usefulness of proposed methodology in this work, water and foods samples were selected to apply this method. Therefore, previously the different steps of sample preparation and clean-up procedure were studied. The analysis was performed by HPLC–GCE-CNTs using the optimized conditions. In order to study the effect of different concentrations of heterocyclic amines in the clean-up procedure, the recoveries were obtained using standard solutions at three concentration levels (0.1, 0.5 and 1 μg/mL).

Regarding water samples, 10 mL of these samples were spiked with standard solutions of the amines and introduced in SPE cartridges. Different types of cartridges preconditioned with 5 mL of MeOH and 10 mL of water were tested, such as Strata-X, Reverse phase Phenomenex and Water Sep-Pak. Only Strata-X cartridges retained properly the analytes; hence this type of cartridge was selected for further analyses. Different volumes (5, 10 and 15 mL) of solution containing MeOH and water (5:95) were used in a cleaning step, being 5 mL of MeOH:H₂O (5:95) adequate to properly remove the interfering substances (inorganic anionic and cationic species, organic matter, and the presence of other potential pollutants). Then, 2 mL of ACN, MeOH, ACN:MeOH (50:50)

solution and the mobile phase used in HPLC-system (acetonitrile:0.05 M ammonium acetate (pH = 7) (25:75)) were used as eluent. Only ACN:MeOH (50:50) completely eluted the retained compound. Therefore, this solution was selected as the eluent solution. Finally, volumes of eluent between 1–5 mL were tested. The higher recoveries of the studied HAAs were obtained using 3 mL of ACN:MeOH (50:50) for all analytes, so this volume was selected as optimum to elute our compounds.

In relation to broth samples, 10 mL of these samples were spiked with standard solutions of the amines and treated with 5 mL of trichloroacetic acid 20%, in order to eliminate proteins. The supernatant was introduced in a Strata-X cartridge preconditioned with 5 mL of MeOH and 10 mL of water. Several binary solutions formed by MeOH and water were used in order to obtain the more interference-free extract (avoiding the interference of carbohydrates, fats, minerals, vitamins, etc). It was achieved using a step washing of cartridge with 3 mL of MeOH:H₂O (15:85) solution. Finally, eluent composition and volume was too optimized. The best result were obtained when 2.5 mL of ACN:MeOH (50:50) were used.

With respect to beef meat sample, a 1 g of the sample was pulverized and homogenized in 10 mL of 1 M NaOH and spiked with standard solutions of the amines. After the treatment of this

Table 2

Recovery values obtained for the analysis of different types of samples by using the proposed method (HPLC/GCE-CNTs).

HAA	Tap water (µg/mL)		River water (µg/mL)		Tablet broth (µg/mL)		Aneto broth (µg/mL)		Beef meat (µg/g)	
	Added	%R	Added	%R	Added	%R	Added	%R	Added	%R
Trp-P-1	0.2	94.4	0.2	96.5	0.05	92.4	0.05	95.5	0.05	93.5
	0.5	95.3	0.5	96.1	0.1	96.3	0.1	96.1	0.1	94.1
	1.0	100.6	1.0	104.8	0.5	95.6	0.5	98.8	0.5	102.8
	3.0	97.7	3.0	99.9	1.0	102.7	1.0	102.9	1.0	99.9
	5.0	101.5	5.0	103.2	5.0	101.5	5.0	97.2	5.0	101.7
Trp-P-2	0.2	93.9	0.2	97.5	0.05	94.9	0.05	97.5	0.05	95.5
	0.5	96.5	0.5	96.1	0.1	100.5	0.1	96.1	0.1	94.1
	1.0	99.7	1.0	104.8	0.5	99.7	0.5	104.8	0.5	96.8
	3.0	103.5	3.0	99.9	1.0	98.5	1.0	99.9	1.0	103.3
	5.0	98.2	5.0	101.5	5.0	103.2	5.0	101.5	5.0	98.5
AαC	0.2	92.9	0.2	92.5	0.05	95.9	0.05	92.5	0.05	97.5
	0.5	94.8	0.5	94.8	0.1	94.8	0.1	94.8	0.1	99.8
	1.0	101.7	1.0	102.4	0.5	101.7	0.5	102.4	0.5	102.4
	3.0	98.4	3.0	95.8	1.0	97.4	1.0	95.8	1.0	97.8
	5.0	97.3	5.0	97.2	5.0	98.3	5.0	97.9	5.0	99.2
H	0.2	93.2	0.2	93.3	0.05	94.2	0.05	93.3	0.05	92.3
	0.5	95.4	0.5	96.3	0.1	94.4	0.1	96.3	0.1	97.3
	1.0	101.8	1.0	97.7	0.5	103.8	0.5	97.7	0.5	95.7
	3.0	102.3	3.0	99.3	1.0	97.3	1.0	99.3	1.0	96.3
	5.0	97.8	5.0	102.8	5.0	101.8	5.0	102.8	5.0	99.8
NH	0.2	94.3	0.2	93.8	0.05	93.9	0.05	93.8	0.05	95.8
	0.5	93.9	0.5	95.1	0.1	96.9	0.1	95.1	0.1	97.1
	1.0	98.9	1.0	98.3	0.5	98.9	0.5	98.3	0.5	103.3
	3.0	102.7	3.0	102.4	1.0	102.7	1.0	102.4	1.0	101.4
	5.0	96.8	5.0	101.7	5.0	98.8	5.0	101.7	5.0	101.2
MeAαC	0.2	95.8	0.2	94.1	0.05	95.8	0.05	94.1	0.05	98.1
	0.5	96.1	0.5	96.9	0.1	103.1	0.1	96.9	0.1	103.4
	1.0	99.4	1.0	96.6	0.5	99.4	0.5	96.6	0.5	97.6
	3.0	102.8	3.0	98.2	1.0	102.8	1.0	98.2	1.0	102.2
	5.0	96.3	5.0	103.1	5.0	99.3	5.0	103.1	5.0	98.6

sample and the addition of 5 mL of trichloroacetic acid 20% (see Section 2.4.), the extract was introduced in a Strata-X cartridge preconditioned with 5 mL of MeOH and 10 mL of water. Different solutions formed by MeOH and water (10:90, 15:85 and 20:80) were tested to obtain the more interference-free extract (the interferences of this type of sample are similar to broth samples). 5 mL of MeOH:H₂O (15:85) solution provided a sample properly cleaned. Finally, eluent composition and volume was optimized and the best result were obtained when 3.0 mL of ACN:MeOH (50:50) were used.

In any case, the optimization of the clean-up procedure was addressed to avoid any interference in the proposed method coming from the matrices of the samples afterwards analyzed. The results of the analyses demonstrated the efficiency of the clean-up procedure incorporated in the whole analytical method here proposed.

3.6. Analytical applications

The proposed method was used to analyse two types of water samples and three foods that were found to contain none of the six studied HAAs. The samples were then spiked with each HAA at variable concentrations in order to study the presence of potential matrix effects. The samples were treated using the procedure optimized in this work (Section 2.4) and analytes were directly separated in isocratic mode with ACN: 0.05 M ammonium acetate pH 7.0 (25:75) as mobile phase. Fig. 4 shows chromatogram obtained for the analysis of beef meat sample natural and spiked with 0.5 µg/mL. As it can be seen, there are not interferences that may affect the determination of studied analytes. Similar results were obtained in all tested samples.

Table 2 shows the results obtained for the determination of the six HAAs in all the tested samples. In this table the values found are

the mean values of ten determinations ($n = 10$). It can be observed that the concentrations added and found were generally agreement and recoveries between 92–105% were obtained. Samples under study were first checked by the proposed method and an independent method (adapted from Kataoka & Kijima, 1997), in order to check the presence of the analytes in these samples. This study demonstrated the absence of HAAs in the samples. Subsequently, these samples were spiked with different concentrations of HAAs and the proposed method for the determination of these compounds was applied. Table 2 reported the recovery values obtained. On the other hand, the analysis of a synthetic control sample containing 1.0 µg/mL of every amine by the proposed and the alternative method demonstrated the comparability of the results when t-test statistical method was applied for a confidence level of 95%.

4. Conclusions

It has been developed an accurate and sensitive method to determine six HAAs in real samples based on the amperometric response of GCE-CNTs after HPLC separation. This work has demonstrated that this electrode exhibits high and good electrocatalytic behavior for these compounds, increasing the sensitivity compared with a conventional glassy carbon electrode. Good linearity, precision and detection and quantification limits were obtained. The percentages of recoveries calculated in all the analysed samples were appropriate for this type of analyses. This method provides a good alternative with respect to conventional process to quantify HAAs in this kind of samples. The simplicity of the procedure to modified available commercially glassy carbon electrodes with MWCNTs opens interesting possibilities to transfer this methodology to routine analytical laboratories working in food safety and control field.

Acknowledgements

Financial support from the Spanish Ministry of Economy and Competitiveness (Project CTQ2013-48411-P) and Junta Comunidades Castilla-La Mancha (Project PEIC-2014-001-P) are gratefully acknowledged.

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