# Determination of Phenolic Antioxidants by HPLC with Amperometric Detection at a Nickel Phthalocyanine Polymer Modified Electrode

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**Abstract**

The suitability of nickel phthalocyanine polymer-coated glassy carbon electrodes for the amperometric detection of the phenolic antioxidants *tert*-butylhydroxytoluene (BHT), *tert*-butylhydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ), after their separation by HPLC under gradient elution conditions, is demonstrated. An applied potential of +0.70 V (vs. Ag/AgCl) was used, which offers the best signal-to-noise ratio and is considerably lower than those reported in the literature with conventional electrodes. A good separation of the four antioxidants was obtained in 17 min. Furthermore, no fouling of the electrode surface was observed when using the polymer modified electrode during the whole working day. Using TBHQ as internal standard, detection limits of 5.5 ng, 7.5 ng and 30 ng (for a 50-μL volume injected) were obtained for BHA, PG and BHT, respectively. As an application, the determination of the antioxidants contained in commercial chewing gum samples was carried out by applying a simple extraction procedure. It was demonstrated that only BHT was present as antioxidant in the samples, in a concentration of $51 \pm 3\ \mu g\ g^{-1}$. Sample linearity and recovery studies, after adding a known amount of BHT to the analyzed samples, confirmed the sutability of the proposed method.

**Keywords:** Polymer modified electrode, Nickel phthalocyanine, Antioxidants

## 1. Introduction

The use of polymer coatings showing electrocatalytic activity to modify electrode surfaces constitutes an interesting approach to fabricate sensing surfaces useful for analytical purposes [1, 2]. In this context, the electrodeposition of polymeric metallophthalocyanines onto solid electrodes has demonstrated to yield chemically modified electrodes which exhibit enhanced electroanalytical responses to numerous important analytes, and possess good mechanical and chemical stability both in aqueous and nonaqueous solvents [3].

The possibility of applying polymeric metallophthalocyanine modified electrodes as indicating electrodes in flowing systems was demonstrated by Baldwin's group with cobalt phthalocyanine electrodes for the determination of various thiols and hydroxylamines [3, 4]. Actually, in our group we have also demonstrated the suitability of a nickel phthalocyanine polymer modified electrode to be used as an amperometric detector under flow-injection conditions [5]. In that work the oxidation of the phenolic antioxidant *tert*-butylhydroxy-anisole (BHA) was used for such a purpose. However, the possibility of employing this polymer modified electrode as an amperometric detector under high performance liquid chromatography (HPLC) conditions, with gradient elution, remained undemostrated.

On the other hand, it is well known that synthetic phenolic antioxidants are commonly added to food products to improve their stability, especially for prevention of lipid oxidation. These phenolic antioxidants, approved by official legislation [6], include *tert*-butylhydroxytoluene (BHT), *tert*-butylhydroxyanisole (BHA) and propyl gallate (PG). Moreover, *tert*-butylhydroquinone (TBHQ) has been also widely employed for this purpose in spite of being not registered nowadays as food additive.

Several methods, usually involving separation steps, have been proposed for the determination of phenolic antioxidants [7]. Regarding high-performance liquid chromatography (HPLC), most applications involved UV detection. Thus, a collaborative study was carried out for the determination of nine phenolic antioxidants in butter oil [8], and a simultaneous determination of antioxidants and preservatives in cosmetics and pharmaceutical preparations by using a linear gradient elution has been reported [9]. Furthermore, Hall et al. made a comparison between capillary electrophoresis and HPLC separations of food grade antioxidants [10]. On the other hand, electrochemical detection has also been used to detect some antioxidants in oil and food samples [11, 12]. Although this detection mode has shown to be a very useful complement to UV detection in the analysis of mixtures which pose interference problems that cannot be resolved spectrally, high potential values have to be applied when using conventional electrodes (glassy carbon) for the detection of BHT. So, applied potentials of +1.0 V and even +1.7 V (vs. Ag/AgCl) have been reported [11, 12], which gives rise to high background currents and, depending on the mobile phase, to an excessive noise. Furthermore, unacceptable baseline drifts usually occur when a gradient elution is employed.

In this article, a nickel(II)-phthalocyanine polymer-coated glassy carbon electrode has been used for the amperometric detection at a moderate applied potential of BHT, BHA, PG and TBHQ, after their separation by HPLC under gradient elution conditions.

## 2. Experimental

### 2.2. Instrumentation and Reagents

A Beckman HPLC system composed of a 125 solvent module, a Model 507e automatic injector, a 168 Diode Array detector (for UV detection), and a Gold System data processor was used. Electrochemical detection was accomplished with a Metrohm

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656 detector equiped with a Metrohm EA 1096 wall-jet cell, and chromatograms were registered on a Metrohm E586 Labograph recorder. Electropolymerization was carried out by using an Autolab PGSTAT 20 System (Ecochemie), and the GPES 3.1 software. A Metrohm 6.0905.010 glassy carbon electrode, a 6.0733.100 Ag/AgCl reference electrode, and a 6.0332.000 Pt counter electrode were used. A Gilson Minipuls peristaltic pump, a P-Selecta ultrasonic bath, a P-Selecta Vibromatic flask shaker and a Heraeus centrifuge were also used.

A Tracer (Teknokroma) 5 µm particle C-18 Nucleosil 120 column (15 × 0.46 cm) kept in a Biorad oven at 40 °C was used in all cases.

A 2% acetic acid (Scharlau) solution in deionized water obtained from a Milli-Q, Millipore system, and methanol (Scharlau, HPLC grade) were employed, degassed prior to use, as mobile phase. A 0.1 mol $L^{-1}$ $HClO_4$ (Panreac) solution was used as the supporting electrolyte for the electrochemical detection.

All antioxidants were from Fluka. Stock solutions of 1000 µg $mL^{-1}$ of each antioxidant in pure methanol were prepared by weighing. More dilute standards were prepared by suitable dilution with pure methanol. Also, stock solutions of these antioxidants were prepared in ethyl acetate. Nickel(II)-4,9,16,23-tetraaminophthalocyanine monomer was purchased from Midcentury Chemical Co.

### 2.2. Procedures

Preparation of nickel phthalocyanine polymer-coated glassy carbon electrodes was carried out by electropolymerization using repetitive cyclic voltammetry between −0.2 and +0.9 V in a dimethyl sulfoxide solution of the monomer, as described previously [5].

### 2.3. Chromatographic Analysis

Due to the different polarities of analytes, gradient elution was used. Eluant A was the 2% acetic acid solution in water, and eluant B was methanol. From 0 to 9 min a linear gradient at a flow rate of 1 mL $min^{-1}$ changes the mobile phase from 90% A, 10% B to 5% A, 95% B. This composition remains for 5 min, and then the mobile phase changes in 1 min to the initial composition, 90% A, 10% B, which is mantained untill the end of run (17 min).

At the exit of the column, a 0.2 mol $L^{-1}$ $HClO_4$ solution, used as the supporting electrolyte for the electrochemical detection, was pumped at a flow rate of 1 mL $min^{-1}$, so that the composition of the mobile phase in the detection cell was always higher than 50% in aqueous phase. Amperometric detection was accomplished at +0.70 V.

The samples analyzed were chewing gum commercial samples purchased in a local supermarket containing an unknown quantity of BHA (E-320) and/or BHT (E-321) as antioxidant.

The sample was cut in small pieces. About 0.5 g (or 2 g) were accurately weighed into a 50-mL centrifuge tube. Then, the sample was spiked with 25 µL of a 1000 µg $mL^{-1}$ TBHQ stock solution in ethyl acetate. Extraction was carried out with 5.0 mL of ethyl acetate. The tube was mechanically shaken overnight. After centrifugation at 3000 rpm for 20 min, a 0.5 mL aliquot of the extract was transferred into a 5 mL glass tube, and evaporated to dryness by passing a $N_2$ stream through. Next, 0.5 mL of methanol were added to the residue and the tube was placed in the ultrasonic bath for 60 s. Volumes of 50 µL were injected without further clean up and relative peak heights (with respect to that of TBHQ, used as internal standard) compared to those of the standards.

## 3. Results and Discussion

The experimental conditions used for the fabrication of the polymer modified electrode, as well as the working medium employed were the same as those optimized previously [5]. In the working medium composed of 0.1 mol $L^{-1}$ $HClO_4$–2% methanol, BHA, BHT, PG and TBHQ showed well-defined oxidation voltammetric peaks (at potentials ranging from 0.33 to 0.58 V) at the Ni-phthalocyanine polymer modified electrode. As an example, Figure 1 shows linear sweep voltammograms for BHA both at the modified electrode and at a bare glassy carbon electrode. As can be deduced from the increase of the peak current and from the shift of the peak potential towards less positive values observed, an increase in the heterogeneous electron transfer rate was produced for the antioxidant oxidation process at the polymeric electrode. Similar results were obtained for the other phenolic antioxidants tested.

Regarding hydrodynamic voltammograms, Figure 2 shows those obtained at the polymeric and at the unmodified electrode from 5.0 mg $L^{-1}$ of BHA in the above mentioned working medium. A wall-jet flow-through cell was used and the carrier solution was also a 0.1 mol $L^{-1}$ perchloric acid-2%, v/v, methanol solution. As can be seen, a well-defined current plateau was obtained at the polymeric electrode exhibiting, as expected, enhanced electroanalytical characteristics with respect to the bare glassy carbon electrode. Again, similar results were achieved for the other phenolic antioxidants. The appropriate potential for the electrochemical detection of the four antioxidants at the polymer modified electrode, following a chromatographic separation, was selected by carrying out a signal-to-background ratio analysis as a function of the applied potential, from the corresponding hydrodynamic voltammograms. Results obtained for BHA are displayed in Figure 3. As can be seen, a potential of +0.70 V allows the best signal-to-background current ratio to be obtained. A similar conclusion was achieved for the other antioxidants. Moreover, this potential is considerably lower than those reported

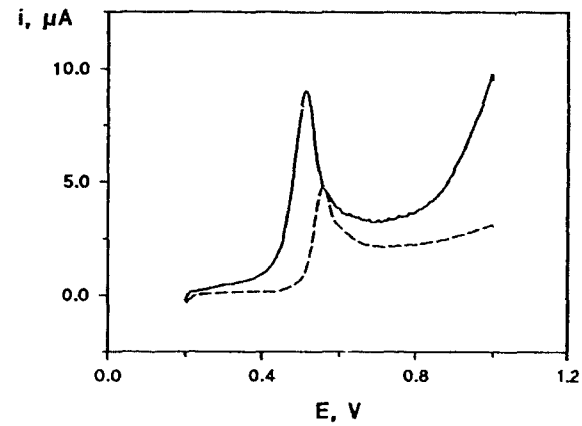


Fig. 1. Linear sweep voltammograms from a 20 mg $L^{-1}$ BHA solution in 0.1 mol $L^{-1}$ $HClO_4$ - 2% methanol (v/v) at a nickel(II)-phthalocyanine polymer modified electrode (—), and at a bare glassy carbon electrode (- - -); $v = 50$ mV $s^{-1}$.

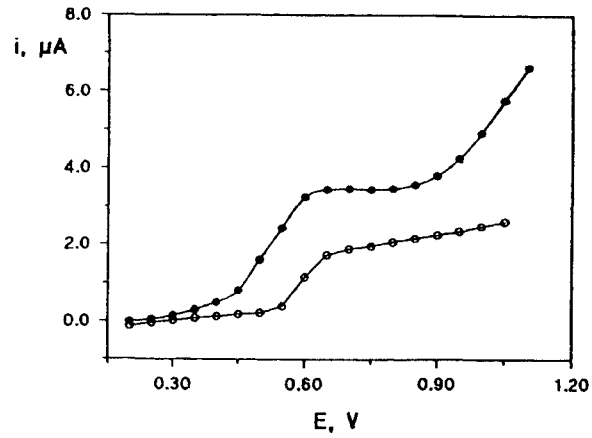


Fig. 2. Hydrodynamic voltammograms of 5.0 mg $L^{-1}$ BHA in 0.1 mol $L^{-1}$ $HClO_4$ - 2% methanol (v/v) at a nickel(II)-phthalocyanine polymer modified electrode (●), and at a bare glassy carbon electrode (○); flow rate 3.3 mL $min^{-1}$.

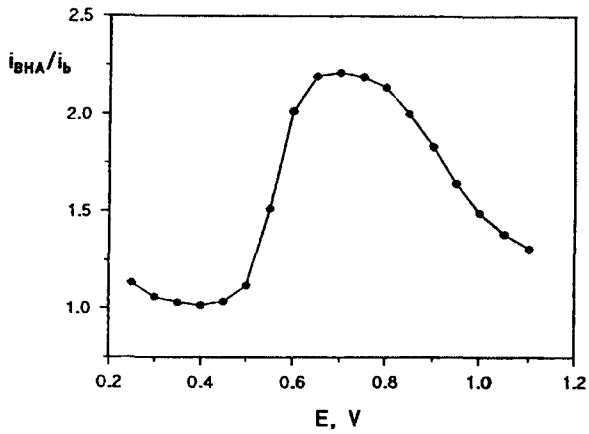


Fig. 3. BHA oxidation current-to-background current ratio as a function of the applied potential obtained from the hydrodynamic voltammogram of 5.0 mg $L^{-1}$ BHA in 0.1 mol $L^{-1}$ $HClO_4$ - 2% methanol (v/v) at a nickel(II)-phthalocyanine polymer modified electrode; flow rate 3.3 mL $min^{-1}$.

in the literature when using conventional electrodes, thus avoiding the drawbacks of the application of extreme potentials mentioned above.

### 3.1. Separation of BHA, BHT, TBHQ and PG

Mixtures of a 2% acetic acid solution in water and methanol were selected as mobile phase for the separation of antioxidants. Several mixtures, ranging from 90 : 10 to 5 : 95 ratios, were tested under isocratic conditions. High methanol contents did not allow the separation of TBHQ and PG, whereas a 90 : 10 mobile phase allowed the four antioxidants to be separated but the retention time of BHT exceeded 50 min. Therefore, a gradient elution was used in spite of having been claimed that this elution can give rise to unacceptable baseline drifts.

Furthermore, as the optimum working medium for the amperometric detection was a 0.1 mol $L^{-1}$ $HClO_4$ medium, a 0.2 mol $L^{-1}$ solution was pumped at the exit of the column into the mobile phase stream at a flow rate of 1 mL $min^{-1}$, so that the final perchloric acid concentration in the amperometric flow cell was 0.1 mol $L^{-1}$.

Figure 4 shows a chromatogram obtained from a mixture containing BHA, BHT and PG, each of them at 2.0 μg $mL^{-1}$ level, and 5.0 μg $mL^{-1}$ TBHQ using the mobile phase gradient displayed also in Figure 4. A 50-μL aliquot was injected. As can be seen, under these conditions a good separation of the four antioxidants was obtained in a quite reasonable time (17 min), which is shorter than most of those reported in the literature [9]. Furthermore, an acceptable baseline was also obtained, being scarcely affected by the gradient elution conditions.

A very important aspect for the practical use of the nickel(II)-phthalocyanine polymer modified electrode is the improvement of this material with respect to the performance of conventional electrodes, in particular of GC electrodes, under chromatographic conditions. It is very well known that some phenolic compounds used as antioxidants have the drawback of fouling of the electrode because of the adsorption of these compounds as well as of their oxidation products onto the electrode surface [13]. This makes a cleaning procedure before each subsequent chromatogram necessary. This important practical problem was avoided when using the polymer modified electrode as the indicating electrode. No fouling problems were observed when using this electrode during the whole working day, thus demonstrating that no adsorption occurred onto the polymer surface. All these practical aspects confirm the potentiality of nickel(II)-phthalocyanine polymer modified electrodes as suitable indicating electrodes in flowing systems.

### 3.2. Calibration Plots and Analytical Characteristics

TBHQ was used as internal standard. It has been excluded from the list of allowed food additives and exhibits similar chemical and electrochemical characteristics as those of the other antioxidants. Thus, the analytical signals employed for the determination of BHA, BHT and PG consisted of the relative peak currents with respect to that of TBHQ. Calibration plots for BHA, BHT and PG in the presence of a constant concentration of TBHQ (5.0 and 0.5 μg $mL^{-1}$ for the upper and lower concentration range considered, respectively) were constructed. Ranges of linearity are shown in Table 1.

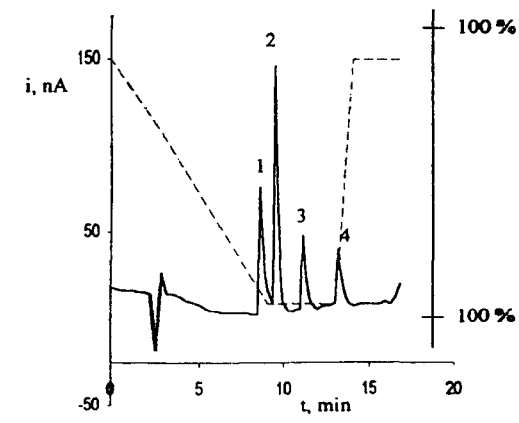


Fig. 4. Chromatogram obtained for a mixture of 2.0 μg $mL^{-1}$ PG (1), BHA (3), and BHT (4), and 5.0 μg $mL^{-1}$ TBHQ (2), with amperometric detection at a nickel phthalocyanine polymer modified electrode. Mobile phase: 2% acetic acid in water (A): methanol (B), gradient elution; $E_{app}$ = +0.70 V; volume injected, 50 μL.

Table 1. Analytical characteristics of the calibration graphs for BHA, BHT and PG using TBHQ as internal standard, obtained by HPLC with amperometric detection at a nickel(II)-phthalocyanine polymer modified electrode: $E_{app}$ = +0.70 V; volume injected: 50 µL; gradient elution as in Figure 3.

| *Antioxidant* | *Range of linearity [µg mL$^{-1}$]* | *Correlation coefficient* | *Slope [µg$^{-1}$ mL]* | *Intercept (relative signal)* | *RSD [%]* | *Detection limit [µg mL$^{-1}$]* |
|---|---|---|---|---|---|---|
| BHA | 0.2–1.0 | 0.9997 | 1.16 ± 0.07 | 0.25 ± 0.04 | 6.5 | 0.11 (5.5 ng) |
| | 2.0–10.0 | 0.9998 | 0.192 ± 0.009 | 0.01 ± 0.04 | | |
| BHT | 2.0–10.0 | 0.998 | 0.12 ± 0.02 | 0.02 ± 0.07 | 7.5 | 0.6 (30 ng) |
| PG | 0.2–1.0 | 0.999 | 0.99 ± 0.08 | 0.28 ± 0.05 | 5.1 | 0.15 (7.5 ng) |
| | 2.0–10 | 0.999 | 0.14 ± 0.02 | 0.00 ± 0.08 | | |

The analytical characteristics calculated from the respective calibration plots are listed in Table 1. Relative standard deviations were calculated from the peak heights ratios from 9 different solutions containing 6.0 µg mL$^{-1}$ of each antioxidant. Each solution was injected by duplicate. The limit of detection was calculated according to the $3s_b/m$ criterion, where $m$ is the slope of the lowest calibration plot, and $s_b$ was estimated as the standard deviation ($n$ = 10) of the relative signal obtained from duplicate injections of solutions containing 0.4 µg mL$^{-1}$ PG and BHA, and 2.0 µg mL$^{-1}$ BHT. Detection limits achieved, about 5 ng for BHA and PG, and 30 ng for BHT, were rather good and demonstrate the validity of the amperometric detection system developed for the determination of the antioxidants at low concentration levels.

### 3.3. Determination of the Antioxidants Contained in Commercial Chewing Gum Samples

As an application of the HPLC with amperometric detection method developed using the nickel(II)-phthalocyanine polymer modified electrode, the determination of antioxidants contained in chewing gum samples was carried out by applying the simple sample pretreatment procedure described in Section 2. These commercial chewing gum samples were labelled to contain an unknown amount of BHA and/or BHT as antioxidants. Figure 5 shows the chromatogram obtained after a 50-µL injection of the sample (0.5 g) extract. As specified in the Section 2., samples were spiked with TBHQ used as the internal standard, so that its concentration in the analyical solution was 5.0 µg mL$^{-1}$. Peak 1 correponded to TBHQ, whereas peak 3 was that of BHT, and peak 2 may be suspected to correspond to BHA in spite of its small height. For comparison purposes, the same chromatogram obtained with UV detection at 288 nm is displayed in Figure 6. As can be observed, in this case TBHQ (peak 1) gave a much higher signal than that of BHT (peak 2). Also, the electrochemical detection at the polymer modified electrode yielded "cleaner" chromatograms from the chewing gum samples extracts than those obtained with UV detection.

In order to ascertain whether the small peak 2 was that of BHA or not, an amount of sample of 2 g was analyzed following the same procedure. Obviously, the analytical solution obtained from the extract of this sample should contain four-fold more BHT and BHA, if this one was present, than for the 0.5 g sample. The chromatogram obtained with amperometric detection is displayed

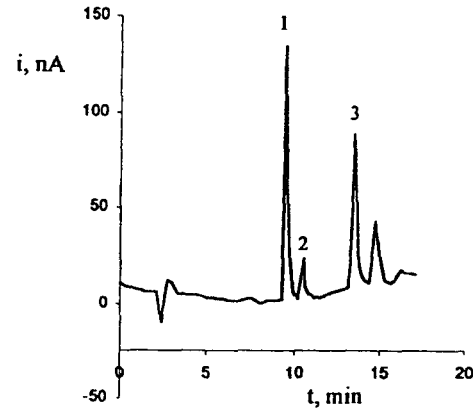


Fig. 5. HPLC-electrochemical detection response with a nickel phthalocyanine polymer modified electrode for a 0.5 g chewing gum sample extract. 1) 5.0 µg mL$^{-1}$ TBHQ used as internal standard; 3) BHT; 2) unknown endogenous compound present in the sample. Other conditions as in Figure 4.

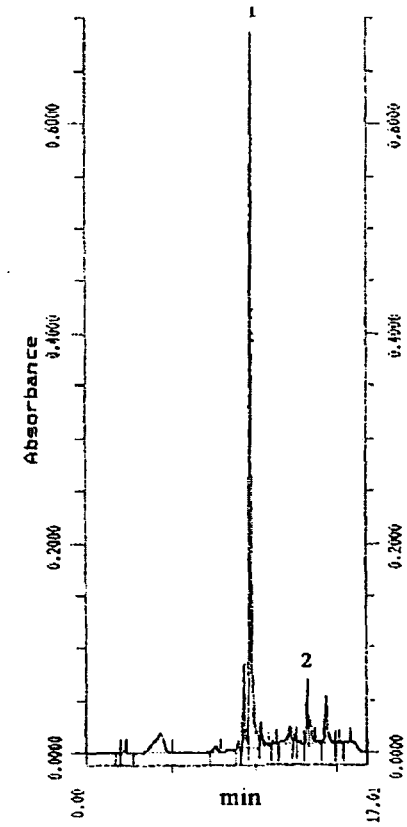


Fig. 6. HPLC-UV detection response for a 0.5 g chewing gum sample extract. 1) 5.0 µg mL$^{-1}$ TBHQ used as internal standard; 2) BHT; $\lambda$ = 288 nm. Other conditions as in Figure 4.

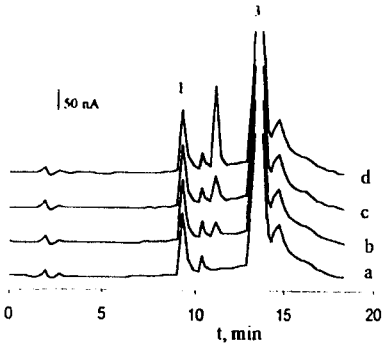


Fig. 7. a) Chromatogram obtained with amperometric detection at a nickel phthalocyanine polymer modified electrode from a 2 g chewing gum sample extract. b)–d) chromatograms obtained after spiking samples with 7.5 μg (b), 15 μg (c), and 45 μg (d) BHA; 5.0 μg $mL^{-1}$ TBHQ used as internal standard. Other conditions as in Figure 4.

in Figure 7, as well as chromatograms corresponding to extracts from samples which were previously spiked with different amounts of a BHA stock solution in ethyl acetate. As can be deduced, peak 2 cannot be assigned to BHA, but it should correspond to another extracted substance present in the chewing gum samples.

Nine determinations of the BHT content in the sample, obtained by interpolation of each relative analytical signal in the corresponding calibration plot, gave a concentration of $51 \pm 3$ μg $g^{-1}$ for a significance level of 0.05. Moreover, sample linearity was tested by plotting the relative analytical signal vs. different amounts of sample (0.0760, 0.1227, 0.2668, 0.5060 and 0.7710 g) and following the same experimental procedure. A linear plot ($r = 0.999$) with a slope of $0.38 \pm 0.03$ and a intercept of $0.09 \pm 0.01$ relative units was obtained in this case.

Furthermore, in order to evaluate the accuracy of this determination, recovery studies were carried out after adding an amount of 25 μg BHT to the 0.5 chewing gum sample, by following the procedure described in the Experimental section. This implies a BHT concentration in the sample of 101 μg $g^{-1}$. Good results were obtained, with an experimental mean concentration, for five determinations, of $98 \pm 4$ μg $g^{-1}$ and a mean recovery of $97 \pm 4$% for a significance level of 0.05, indicating that the methodology proposed is suitable for the determination of the antioxidants contained in this type of samples.

## 4. Conclusions

All the aforementioned results demonstrate the suitability of nickel(II)-phthalocyanine polymer modified electrodes for use as electrochemical detection systems in HPLC. They allow the determination of the antioxidants BHT, BHA and PG, using TBHQ as internal standard, with an adequate sensitivity and at moderate applied potentials. Moreover, the polymer coating prevents the fouling of the electrode surface. Furthermore, the time of analysis of the antioxidants is rather short and, when the methodology proposed is applied to the analysis of chewing gum samples, the chromatograms of the corresponding sample extracts are cleaner than those obtained with UV detection.

## 5. Acknowledgements

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