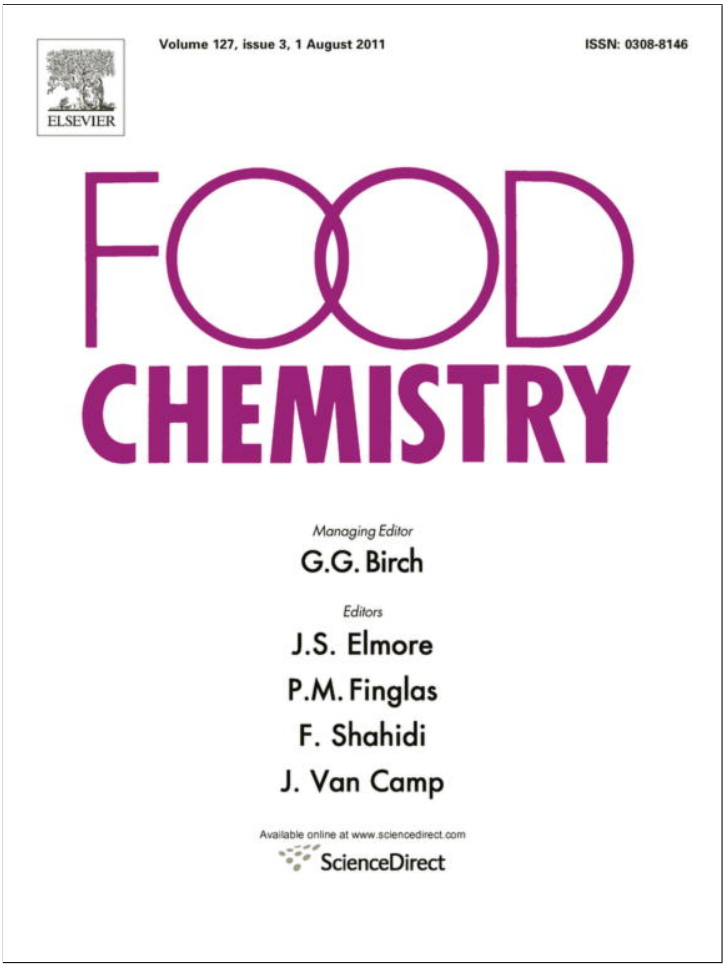

Food Chemistry 127 (2011) 1361–1369

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Qualitative and quantitative electroanalysis of synthetic phenolic antioxidant mixtures in edible oils based on their acid–base properties

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ARTICLE INFO

Article history:
Received 5 March 2010
Received in revised form 7 January 2011
Accepted 24 January 2011
Available online 31 January 2011

Keywords:
Synthetic phenolic antioxidants
Square wave voltammetry
Edible oils
Standard addition method

ABSTRACT

A simple electroanalytical method using square wave voltammetry at a Pt band ultramicroelectrode to perform a qualitative and quantitative analysis of different synthetic antioxidant mixtures permitted by official regulations in edible oils is proposed. The methodology was based on the comparison of voltammetric signals obtained in acetonitrile + 0.1 M $(C_4H_9)_4NF_6P$ with those recorded in the same reaction medium when different aliquots of $(C_4H_9)_4NOH$ were added to allow a qualitative differentiation between antioxidants. Firstly, studies on solutions prepared from commercial reagents were carried out. Then, the results obtained were transferred to the analysis of a real matrix, i.e., an edible olive oil. From real samples spiked with a known amount of different synthetic antioxidant mixtures, we could deduce the presence of these antioxidants by comparing results obtained in the neutral medium with those obtained after the successive addition of base. The standard addition method was used to quantify the individually spiked synthetic antioxidants in the real sample. Recovery percentages were between 88% and 118%. The reproducibility was 1.5%, 3.1%, 4.1% and 4.1% in ACN + 0.1 M TBAHFP and 1.5%, 4.6%, 6.6% and 2.5% in Bz/EtOH (1:2) + 0.1 M H_2SO_4 for TBHQ, BHA, BHT and PG, respectively. The repeatability was 1% for PG in both media. These parameters show a good system performance.

1. Introduction

The use of unauthorized antioxidants in food constitutes an illegal action. Therefore, the analysis of antioxidants is a critical control point to ensure quality and to avoid adulterations, which are forbidden by law according to the Code of Ethics for International Trade in Food (Codex Alimentarius., 2009) and other regional regulations (Código Alimentario Argentino, 2009).

Tert-butyl hydroxyanisole (BHA), tert-butyl hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ) and propyl gallate (PG) are the commonest synthetic phenolic antioxidants used in edible oils or lipid-based food in order to prevent oxidative rancidity (Rajalakshmi Narasimhan, 1996). Their chemical structures are the following:

Official regulations do not permit the use of certain synthetic antioxidant mixtures such as TBHQ with gallates. However, the regional regulation (Código Alimentario Argentino, 2009) permits other mixtures in limited ratios such as BHA with BHT and/or TBHQ at a total concentration of 200 ppm as well as PG with BHA and/or BHT at a total concentration of 200 ppm and no more than 100 ppm of gallates. In addition, the ternary mixtures BHA + BHT + TBHQ and BHA + BHT + PG at a total concentration of 200 ppm are also permitted (Código Alimentario Argentino, 2009).

Analytical methods, including spectrophotometry (Viplava Prasad, Divakar, Hariprasad, Sastry, 1987), gas chromatography (González, Gallego, Valcárcel, 1999), and HPLC (Perrin Meyer, 2002; Sin, Wong, Mak, Sze, Yao, 2006) for analysis of a wide variety of synthetic antioxidants in different types of food have been reported. Besides, the chemometric analysis to determine mixtures of synthetic antioxidants has been used (Galeano Díaz, Guiberteau Cabanillas, Alexandre Franco, Salinas, Viré, 1998; Ni, Wang, Kokot, 2000). Both, the detection and quantification of extra-virgin olive oil adulteration with different edible oils using mid-infrared (IR) spectroscopy with chemometrics (Gurdeniz Ozen, 2009) and the adulteration of refined olive oil with refined hazelnut oil employing NMR spectroscopy and multivariate statistical analysis (Agiomyrgianaki, Petrakis, Dais, 2010) have been recently described. On the other hand, the advantages of synthetic antioxidant electroanalytical determinations have been claimed (Agui, Reviejo, Yañez-Sedeño, Pingarrón, 1995; Ceballos Fernández, 2000a; Ceballos Fernández, 2000b; Galeano Díaz et al., 1998; McBride Evans, 1973). However, it is known that overlapping of electroanalytical responses in mixtures of synthetic phenolic antioxidants

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doi:10.1016/j.foodchem.2011.01.102

is sometimes strong, generally preventing their simultaneous quantification.

Pulse voltammetric techniques, mainly square wave voltammetry (SWV) combined with ultramicroelectrodes (UME) have been proposed to obtain highly reproducible signals and to improve both speed and sensitivity compared with other electrochemical techniques. Therefore, the use of UME and SWV appears as a good alternative for electrochemical analysis (Ceballos Fernández, 2000b; Osteryoung, 1990). The electrochemical determination of a mixture containing BHA, BHT and TBHQ using different techniques, pH, working electrodes, and distinct supporting electrolytes has been recently used for simultaneous determination of them without chemometric approaches or prior pretreatments (dos Santos Raymundo, Marques da Silva Paula, Franco, Fett, 2007). However, the synthetic phenolic antioxidant acid–base properties have not yet been used to obtain a qualitative differentiation between them.

In this work, we propose a simple electroanalytical method using SWV at Pt band UME based on the analysis of voltammetric signals of synthetic antioxidant mixtures spiked in an olive oil matrix. Antioxidants were extracted with acetonitrile (ACN). Then, their electrochemical responses were studied in ACN + 0.1 M $(C_4H_9)_4NF_6P$ (TBAHFP) and compared with changes produced by successive additions of $(C_4H_9)_4NOH$ (TBAOH). Firstly, we used commercial reagents in our studies in order to characterise qualitatively the presence of only one antioxidant, or mixtures of them, in the reaction media. Secondly, we transferred these results to the analysis of a real matrix, i.e. an edible olive oil with no synthetic antioxidants added (according to the manufacturer's label) spiked with mixtures of different synthetic antioxidants at known amounts. Besides, the standard addition method (Skoog, Holler, Nieman T. A., 2001) was used to quantify the individually spiked synthetic antioxidants in the real matrix.

2. Experimental

2.1. Chemicals

BHA, BHT, PG, and TBHQ (Sigma Chemical Co., St. Louis, MO), TBAHFP, $(C_2H_5)_4NClO_4$ (TEAP), and TBAOH (Fluka, electrochemical grade), benzene (Bz, Sintorgan, HPLC), ethanol (EtOH, Merck p.a.), and H_2SO_4 (Merck p.a.) were used as received. ACN (Sintorgan, HPLC-grade) was kept over 3 Å molecular sieves during 48 h prior to use. Then, it was used without further purification. We purchased the olive oil at a local supermarket, with no added synthetic antioxidants indicated on the label.

Oil samples were dissolved in a Bz + EtOH binary mixture (1:2) in 0.1 M H_2SO_4. Studies on edible olive oil samples spiked with synthetic antioxidants, after extraction steps with ACN, were carried out following a methodology previously described (Ceballos Fernández, 2000b). The only modification performed was that the sonicating step for 4 min was replaced by a more efficient vortex step of 2 min. Then, the mixture was transferred to an ultrasound bath during other 2 min. This procedure allowed us to improve oil extraction steps.

2.2. Materials

A two-compartment Pyrex cell using a three-electrode configuration with an AutoLab PGSTAT 12 potentiostat, controlled by GPES 4.9 electrochemical software, Eco-Chemie, Utrecht (The Netherlands) was used to perform SWV experiments. The characteristic parameters of SW voltammograms were a frequency (f) of 25 Hz, square wave amplitude (ΔE_{sw}) of 0.050 V, and a staircase step height (ΔE_s) of 0.005 V.

A carbon fiber disk UME (= 11 μm) and a Pt band UME constructed in our laboratory were used as working electrodes. The pretreatment used for each one was the same as previously reported (Ceballos Fernández, 1995; Ceballos Fernández, 2000a). The reference electrode was an aqueous saturated calomel electrode (SCE) and the counter electrode was a platinum foil of large area (approx. 2 cm^2).

A HP model 8463 spectrophotometer was used for the determination of antioxidant acid equilibrium constants following a procedure reported in the literature (Molina, Zón, Fernández, 1998).

The standard addition method was used to determine the recovery percentages from olive oil samples spiked with synthetic antioxidants in both the olive oil matrix and after extraction steps with ACN. All measurements were carried out at a temperature of 22.0 ± 0.5 °C.

2.3. Calculation of the Pt band electrode area

Linear relationships between the net peak current ($I_{p,n}$) from SWV measurements and the concentration of the electro-active species were found for all individual antioxidants in the concentration range from 20 to 200 ppm. The Pt band UME showed a very good sensitivity (see Section 3.2.2.). Its electrochemical area was determined by chronoamperometric measurements of ferrocene (Fc) in ACN + 0.1 M TEAP at 20.0 °C by assuming a Fc diffusion coefficient of 2.26×10^{-5} cm^2 s^{-1} (Zón, Moressi, Sereno, Fernández, 1994). Szabo, Cope, Tallman, Kovach, and Wightman (1987) have proposed that, for a band UME of width w, its chronoamperometric response can be approximated at long times (t) with that corresponding to a hemicylinder electrode of equal area and with an equivalent radio of $w/4$ (Eq. (1)).

$$\frac{i(t)}{nFDc_0 l} = \frac{\pi e^{\frac{2\sqrt{\pi\tau}}{5}}}{4\sqrt{\pi\tau}} + \frac{\pi}{\ln\left[(64e^{-\gamma}\tau)^{1/2} + e^{5/3}\right]}; \tau > 2/5 \quad (1)$$

where $i(t)$ is the amperometric current response; D and c_O are the diffusion coefficient and the bulk concentration of electro-active species, respectively; l is the band length; $\tau = Dt/w^2$; γ is a constant ($\gamma = 0.5772156\ldots$), and the other terms have their usual meaning.

The MatLab program (version 7.0) was used for fitting Pt band UME amperometric responses in order to determine its electrochemical area. The goodness of the fit was evaluated by using the R-square (R^2) and the root mean squared error (RMSE) statistics parameters. For other calculations, we used the Origin program, version 7.5.

Therefore, experimental chronoamperometric responses were fitted with the theoretical equation proposed (Eq. (1)). The fitting was very good, with $R^2 = 0.9853$ and RMSE = 5×10^{-8} A. An electrochemical area of 1.3×10^{-3} cm^2 was calculated.

3. Results and discussion

3.1. Studies performed in solutions of commercial reagents

Net currents (I_n) of SW voltammograms recorded for BHA (A), BHT (B), TBHQ (C) and PG (D) in ACN + 0.1 M TBAHFP at a given synthetic antioxidant concentration ($c^*_{A_{ox}}$) in the absence (line 1) and in the presence (lines 2 and 3) of different aliquots of TBAOH are shown in Fig. 1. Voltammetric responses of BHT (net peak potential, $E_{p,n} = 1.24$ V) and PG ($E_{p,n} = 1.25$ V) strongly overlap in this medium (compare Figs. 1B and D, lines 1). A similar behaviour was found for voltammetric responses of BHA ($E_{p,n} = 1.04$ V) and TBHQ ($E_{p,n} = 0.96$ V), as can be observed in Figs. 1A and C (lines 1). Interesting changes were observed when a base (TBAOH) was added to the solution. A diminution in the current intensity of these

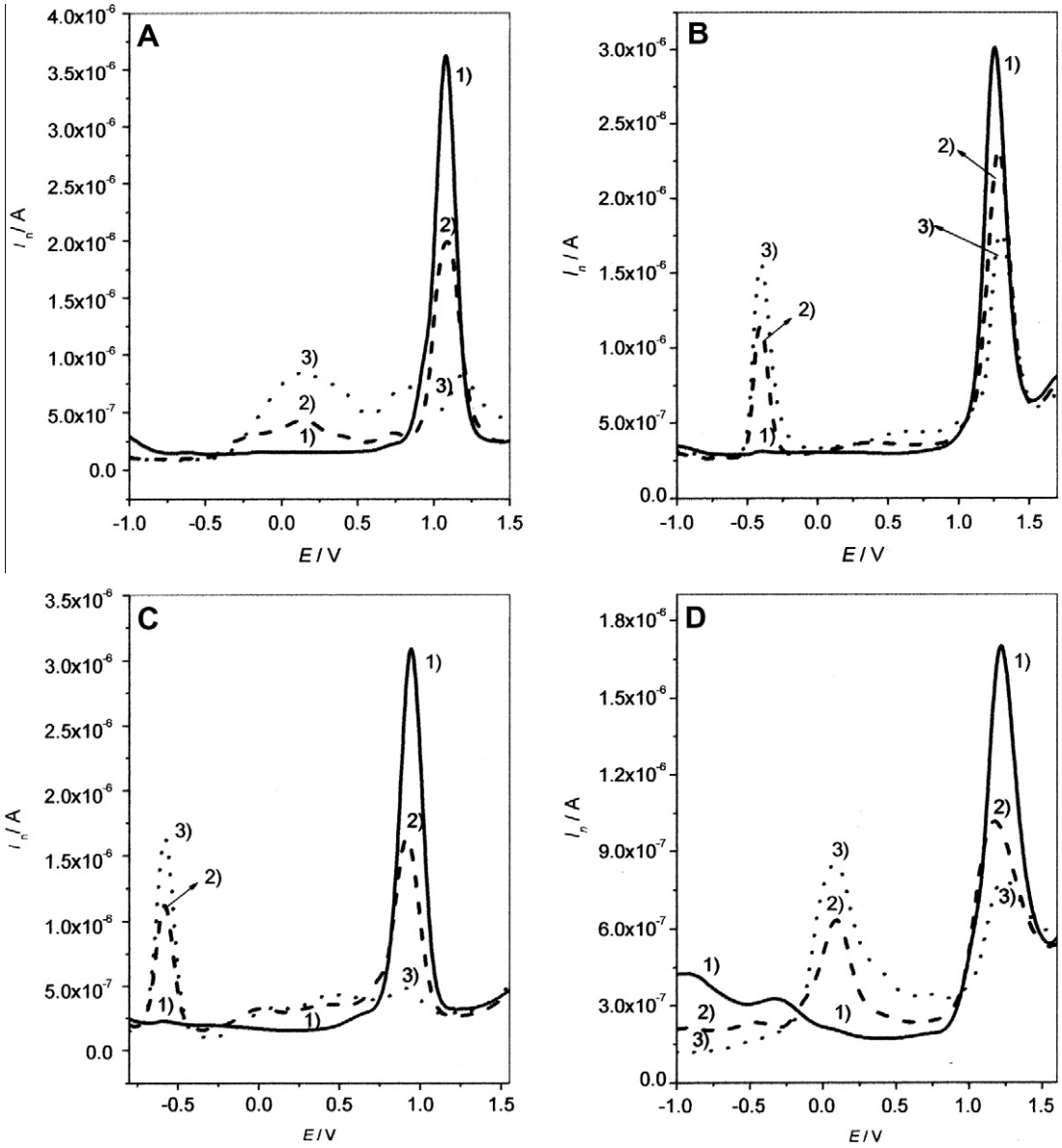

Fig. 1. Square wave voltammograms of BHA (A), BHT (B), TBHQ (C) and PG (D) in ACN + 0.1 M TBAHFP in the absence (solid lines, 1) and in the presence (dash lines, 2 and dot lines, 3) of TBAOH. $c^*_{A_{ox}} = 1.10 \times 10^{-3}$ M, $c^*_{BHT} = 9.15 \times 10^{-4}$ M, $c^*_{TBHQ} = 1.25 \times 10^{-3}$ M and $c^*_{PG} = 9.03 \times 10^{-4}$ M. c^*_{TBAOH} = (A) 8.30×10^{-4} M and 2.21×10^{-3} M for lines 2 and 3, respectively; (B) 4.15×10^{-4} M and 8.30×10^{-4} M for lines 2 and 3, respectively; (C) 5.54×10^{-4} M and 8.30×10^{-4} M for lines 2 and 3, respectively and D) 5.54×10^{-4} M and 1.11×10^{-3} M for lines 2 and 3, respectively. Working electrode: a Pt band UME. Reference electrode: SCE. $\Delta E_{sw} = 0.050$ V, $\Delta E_s = 0.005$ V and $f = 25$ Hz.

voltammetric peaks was observed in the presence of the base as well as the emergence of new peak/s at potentials less positive than the original voltammetric peak. The presence of phenoxide ion/s because of acid–base equilibria established in solution (Evans, Jiménez, Kelly, 1984; Hammerich Svensmark, 1991) would be the main responsible species for those new peaks.

The behaviour of BHA in the presence of the base was more complex than that found for the other antioxidants. In spite of BHA having only one phenolic –OH in its chemical structure, it is well known that commercial BHA is a mixture of two isomers, 2-tert-butyl-4-hydroxyanisole (2-BHA) and 3-tert-butyl-hydroxy-anisole (3-BHA), with a 90% of the isomer 3-BHA (Rajalakshmi Narasimhan, 1996). The acidity of 3-BHA can be predicted to be slightly higher than the acidity of 2-BHA by considering that the –OH phenolic is closer to the tert-butyl group in the 2-isomer than in the 3-isomer (Morrison Boyd, 1990). Therefore, two new peaks were developed in the potential range from −0.45 V to 0.54 V when the base was added, with $E_{p,n} \cong 0.16$ and 0.42 V, respectively. Another voltammetric peak centered at about $E_{p,n} \cong 0.78$ V was also observed when the amount of added base increased. PG has three phenolic –OH in its molecular structure. A new voltammetric peak centered at $E_{p,n} \cong 0.09$ V appeared in the presence of the base showing a post-shoulder as the base concentration increased. Moreover, the voltammetric signal in the potential range between −1.00 and 0.30 V decreased under these experimental conditions. BHT is a weak monoprotic acid. Only one new peak ($E_{p,n} = -0.40$ V) was observed in the presence of the base, which increased as the base concentration increased. TBHQ has a hydroquinone species in its molecular structure, being therefore a weak diprotic acid. However, the acidity of its two phenolic –OH can be predicted to be slightly different due to the farther or closer proximity of –OH groups to the $-C(CH_3)_3$ substituent. A new main peak centered at $E_{p,n} = -0.58$ V and two almost negligible peaks (at 0.00 and 0.50 V, respectively) appeared as the base concentration increased.

The most common synthetic antioxidant mixtures added to vegetable edible oils are the following: BHA + BHT, BHA + TBHQ, BHA + PG, PG + BHT, BHA + BHT + TBHQ and BHA + BHT + PG. Hence, we have also studied the effect of these mixtures on the voltammetric responses after the addition of different aliquots of TBAOH in the reaction medium. SW voltammograms of BHA + BHT (A), BHA + TBHQ (B), BHA + PG (C) and PG + BHT (D) mixtures in the absence (lines 1) and in the presence of different concentrations of the base (lines 2 and 3) are shown in Fig. 2. Two net SW peaks were found for the BHA + BHT mixture in the absence of the base, with $E_{p,n}$ centered at about 1.08 and 1.35 V, respectively (Fig. 2A, line 1). The current intensity of these peaks decreased as a new peak at a $E_{p,n} = -0.40$ V was observed, whose intensity increased as the TBAOH concentration increased. This peak shows the presence of BHT in the mixture. In addition, new voltammetric signals were developed in the potential range from 0.00 to 0.84 V in the presence of the base, with peaks centered at $E_{p,n} \cong 0.16$, 0.42 and 0.74 V, respectively. These peaks are characteristic of the presence of BHA in the presence of the base. On the other hand, only one main SW voltammetric peak was found for the BHA + TBHQ mixture in the absence of the base (Fig. 2B, line 1), with $E_{p,n} = 1.02$ V. This behaviour can be explained by considering that the SW signals of BHA and TBHQ were strongly overlapped (see Fig. 1A and C). Both, a diminution in the peak current of this peak and a new peak centered at $E_{p,n} = -0.60$ V were observed when a small amount of the base was added (Fig 2B, line 2). In addition, other two new peaks were observed when the TBAOH concentration was increased. These new peaks centered at $E_{p,n} \cong 0.13$ and 0.74 V (Fig. 2B, line 3) can be assigned to the corresponding BHA phenoxide ions while the peak at $E_{p,n} = -0.60$ V corresponds to the TBHQ phenoxide ion as previously explained. The origin of the peak at −0.20 V cannot be assigned with precision yet,

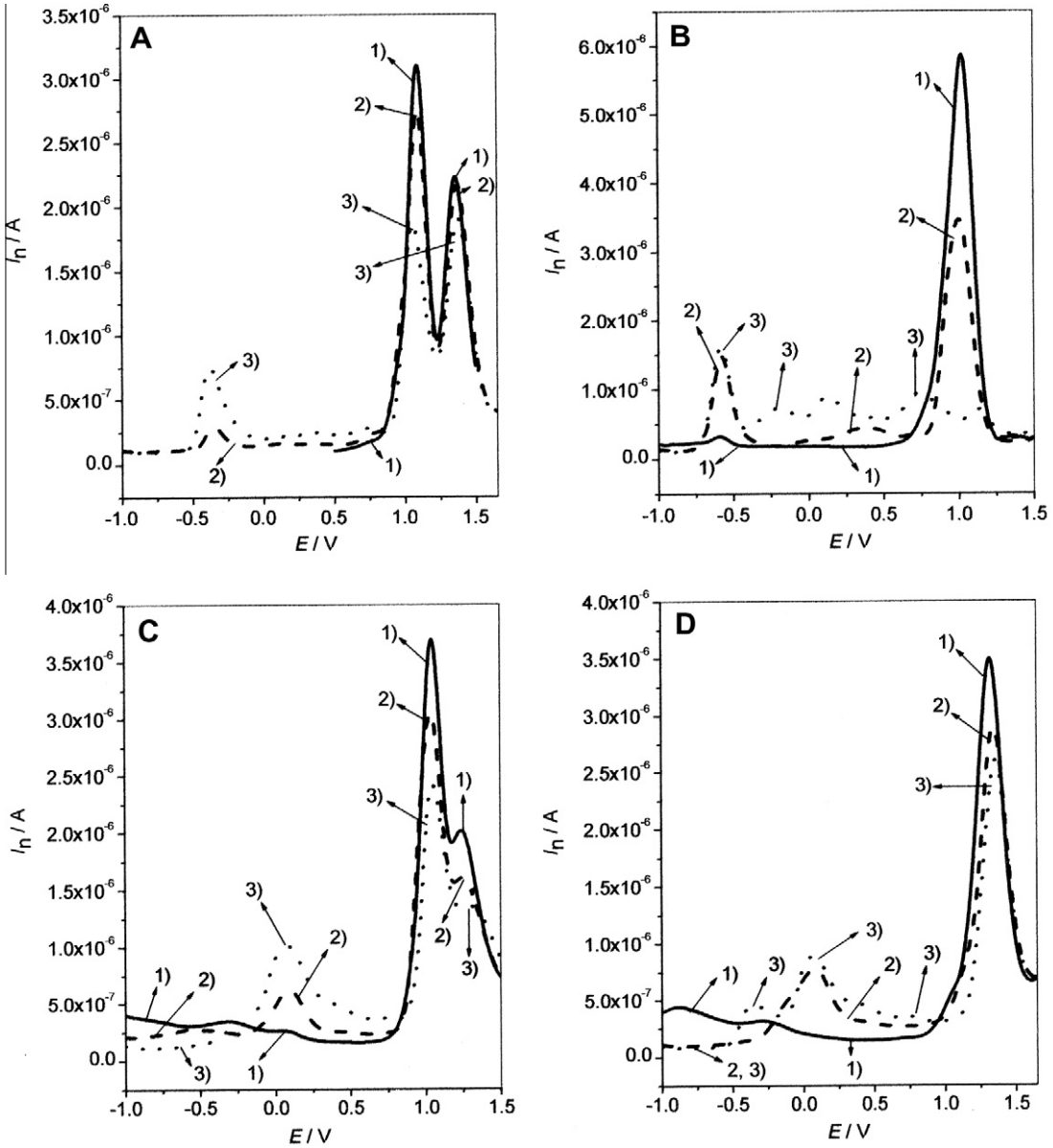

Fig. 2. Square wave voltammograms of BHA + BHT (A), BHA + TBHQ (B), BHA + PG (C) and PG + BHT (D) mixtures in ACN + 0.1 M TBAHFP in the absence (solid lines, 1) and in the presence (dash lines, 2 and dot lines, 3) of different aliquots of TBAOH. Antioxidant concentrations in all mixtures were: $c^*_{BHA} = 1.10 \times 10^{-3}$ M, $c^*_{BHT} = 9.13 \times 10^{-4}$ M, $c^*_{TBHQ} = 9.60 \times 10^{-4}$ M and $c^*_{PG} = 9.03 \times 10^{-4}$ M. c^*_{TBAOH} = (A) 2.77×10^{-4} M and 8.30×10^{-4} M for lines 2 and 3, respectively; (B) 1.38×10^{-3} M and 2.77×10^{-3} M for lines 2 and 3, respectively; (C) 8.04×10^{-4} M and 1.52×10^{-3} M for lines 2 and 3, respectively and D) 1.24×10^{-3} M and 2.08×10^{-3} M for lines 2 and 3, respectively. Other experimental conditions are the same as Fig. 1.

although it can be associated to the simultaneous presence of BHA and TBHQ in solution (see below for the BHA + BHT + TBHQ mixture). The peak at 0.42 V for an intermediate base concentration can be assigned to BHA (see Fig. 1A).

The SW voltammograms of BHA + PG mixture in the absence of the base showed two voltammetric peaks centered at $E_{p,n} \cong 1.05$ and 1.25 (Fig. 2C, line 1), which can be assigned to BHA and PG, respectively. Under these experimental conditions, low voltammetric signals in the potential range from −1.00 to about 0.30 V were due to the presence of PG in the mixture (compare Fig. 2C, line 1 with Fig. 1D, line 1). As the base concentration increased both peaks at higher potentials and signals in the range between −1.00 and 0.30 V decreased. In addition, a wide new peak appeared and increased as the base concentration increased, with a $E_{p,n} \cong 0.07$ V. This peak denotes the presence of PG in the mixture. Peaks of low intensity at about 0.42 and 0.74 V denote the presence of BHA. Their current peaks increased as the base concentration increased. It is also important to note that the PG signal at 0.07 V increased much more rapidly with the base addition than the BHA signals. This phenomenon may be explained by considering that PG acidity is higher than BHA acidity (see below).

The SW voltammetric response of PG + BHT mixture in the neutral medium showed a well-defined peak at $E_{p,n} \cong 1.33$ V as a consequence of the strong overlapping of the individual voltammetric responses of PG and BHT (Fig. 2D, line 1). In this case, voltammetric signals in the potential range from −1.00 to about 0.13 V were also found, which can be assigned to the presence of PG in the mixture, as previously observed for BHA + PG mixture. A diminution of these voltammetric signals and the main original oxidation peak was observed as the base concentration increased (Fig. 2D, lines 2 and 3). Under the latter experimental conditions, new voltammetric peaks appeared in the potential range from −0.55 to 0.93 V, with two main peaks centered at about −0.40 and 0.07 V, indicating the presence of BHT and PG phenoxide ions, respectively.

Fig. 3A shows SW voltammetric response of BHA + BHT + TBHQ mixture. Two main oxidation peaks, with $E_{p,n} \cong 1.05$ and 1.33 V were observed in the absence of the base (Fig. 3A, line 1), which could be assigned to the presence of BHA + TBHQ and BHT, respectively. These peaks decreased, and a new well-defined peak at $E_{p,n} \cong -0.58$ V, as well as voltammetric signals centered at about 0.10 and 0.40 V when an aliquot of the base was added, indicate the presence of TBHQ and BHA, respectively. Conversely, the peak at $E_{p,n} \cong -0.58$ V and new peaks with $E_{p,n} \cong -0.40$, −0.21 and 0.74 V, and a more pronounced diminution in peaks at potentials greater than 1.00 V were found as the base concentration increased (Fig. 3A, line 3). Peaks at $E_{p,n} \cong -0.58$ and 0.74 V clearly indicate the presence of TBHQ and BHA, respectively, in the mixture. As mentioned above (Fig. 2B, line 3), the peak at $E_{p,n} \cong -0.21$ V agrees with the presence of BHA and TBHQ and it was observed when a base concentration similar to the antioxidants was added.

Fig. 3B shows SW voltammograms of another possible synthetic antioxidant ternary mixture (BHA + BHT + PG) in the absence (line 1) and in the presence of base (lines 2–4). Two main oxidation peaks, with $E_{p,n} \cong 1.05$, and 1.33 V were clearly observed in the absence of the base, and weak voltammetric signals were evident in the potential range from −1.00 to 0.30 V (Fig. 3B, line 1). Peaks at $E_{p,n} \cong 1.05$ and 1.33 V correspond to voltammetric responses of BHA and the BHT + PG mixture, respectively. Weak signals in the region between −1.00 and 0.30 V agree with the PG presence. When small aliquots of TBAOH were added, the main oxidation peaks decreased and a new peak at $E_{p,n} \cong 0.07$ V appeared (Fig. 3B, lines 2 and 3). At a greater base concentration, the diminution of peaks in the potential region from 0.80 to 1.55 V was more pronounced and a sigmoid signal in the potential range between −0.50 and −0.25 V as well as two new peaks at $E_{p,n} \cong 0.42$ and 0.74 V were observed. The signal at 0.07 V is characteristic of PG and those at 0.42 and 0.74 V are characteristic of the presence of BHA in the mixture. Moreover, the sigmoid signal could be associated with BHT phenoxide ion, whose voltammetric response may not be well defined. Consequently, BHT and PG ion phenoxide SW peaks in this complex mixture would be slightly overlapped.

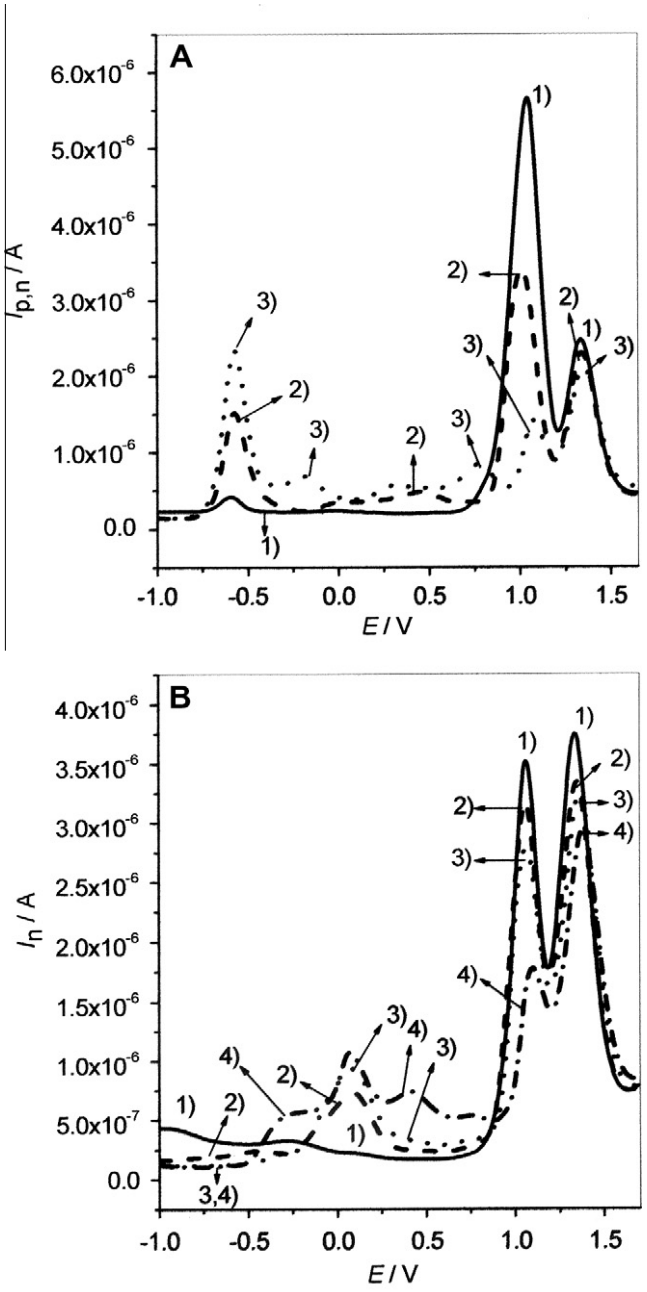

Fig. 3. (A) Square wave voltammograms of BHA + BHT + TBHQ mixture in ACN + 0.1 M TBAHFP in the absence (solid line, 1) and in the presence (dash line, 2 and dot line, 3) of different aliquots of TBAOH. $c^*_{TBAOH} = 1.38 \times 10^{-3}$ M (line 2) and 2.49×10^{-3} M (line 3). (B) SW voltammograms of BHA + BHT + PG mixture in the same reaction medium as a) in the absence (solid line, 1) and in the presence of different amounts of base (dash line, 2; dot line, 3 and dash dot line, 4). $c^*_{BHA} = 1.00 \times 10^{-3}$ M, $c^*_{BHT} = 9.13 \times 10^{-4}$ M, $c^*_{TBHQ} = 9.60 \times 10^{-4}$ M and $c^*_{PG} = 9.24 \times 10^{-4}$ M; $c^*_{TBAOH} = 8.30 \times 10^{-4}$ M (line 2), 1.24×10^{-3} M (line 3) and 2.63×10^{-3} M (line 4). Other experimental conditions are the same as Fig. 1.

The results described above would allow inferring qualitatively which of all possible mixtures would be present in edible oils. If SW voltammograms in the neutral medium present only one

voltammetric peak, the only possible synthetic antioxidant mixtures would be: BHA + TBHQ and PG + BHT, which can be distinguished by considering that their $E_{p,n}$ in neutral medium would be 1.05 and 1.33 V, respectively. Moreover, the BHA + TBHQ mixture could be verified by the emergence of a peak at −0.58 V after base addition, characteristic of TBHQ phenoxide ion, as well as three peaks of BHA phenoxide ions in the potential region from −0.40 to 1.00 V. On the other hand, the PG + BHT mixture would show a BHT phenoxide ion peak at −0.40 V and a wide PG phenoxide ion peak at about 0.07 V in the presence of TBAOH.

In addition, if SW voltammograms of mixtures in the neutral medium show two voltammetric peaks in the potential range from 0.70 to 1.55 V, the only possible mixtures would be: BHA + BHT, BHA + PG, BHA + BHT + TBHQ and BHA + BHT + PG.

BHA + BHT could be easily distinguished from BHA + PG in the presence of TBAOH because the first mixture would show a well-defined voltammetric peak at about −0.40 V while the second mixture would exhibit a wide voltammetric peak at about 0.07 V.

Moreover, we estimated the apparent acid dissociation constant/s (K_{ai}) for these synthetic phenolic antioxidants through UV–visible spectrophometric measurements at different pH values following a methodology previously described by us (Molina et al., 1998). Therefore, we calculated $K_a = (9.01 \pm 0.01) \times 10^{-13}$ for BHA; $K_{a1} = (9.55 \pm 0.01) \times 10^{-9}$, $K_{a2} = (8.98 \pm 0.01) \times 10^{-11}$ and $K_{a3} = (5.17 \pm 0.01) \times 10^{-12}$ for PG; $K_{a1} = (1.48 \pm 0.01) \times 10^{-9}$ and $K_{a2} = (1.38 \pm 0.01) \times 10^{-12}$ for TBHQ and $K_a = (3.47 \pm 0.01) \times 10^{-14}$ for BHT. On the basis of these results, the acidity of these compounds is as follows: PG > TBHQ > BHA > BHT. These results are in very good agreement with the rate diminution of peak currents of the main antioxidant peaks in the neutral medium as the base addition was increased, even though the reaction medium was different.

3.2. Studies in real samples

3.2.1. Blank solution in olive oil

Very weak voltammetric responses were found in the potential range from about 0.30 to 1.00 V when an olive oil aliquot of 1.5 mL was dissolved in Bz/EtOH (1:2) + 0.1 M H_2SO_4 solvent mixture. SW voltammograms were recorded at a carbon fiber disk UME in this medium. As it is well established, the employment of Pt electrodes in an aqueous acid medium precludes any electrochemical antioxidant study because of hydrogen ion discharge and Pt oxide formation (Sawyer, Sobkowiak, Roberts, 1995). The weak voltammetric responses found at carbon fiber disk UME correspond to natural antioxidants (tocopherols) present in olive oil. However, it is well known that α-tocopherol is the most important and active natural antioxidant present in olive oil (Galeano Díaz, Durán Merás, Guiberteau Cabanillas, Alexandre Franco, 2004). A little increase in the voltammetric signal at potentials higher than 1.00 V could correspond to other substances present in the olive oil. However, no SW voltammetric signal corresponding to synthetic antioxidant was found, which is in agreement with the manufacturer's label. SW voltammograms obtained in ACN + 0.1 M TBAHFP at Pt band UME before and after extraction steps when the oil sample, without any synthetic antioxidant added, showed a similar blank to that formerly described for the olive oil in the acidic reaction medium at a carbon fiber disk UME.

3.3. Recovery percentages

Pools of three samples of olive oil spiked with different concentrations of antioxidant (TBHQ, samples 1–3, $n = 5$ each one by triplicate), BHA (samples 4–6, $n = 6$ each one by triplicate) and BHT (samples 7–9, $n = 7$ each one by triplicate) and five samples for PG (samples 10–12, $n = 7$ each one by triplicate) were used to obtain the recovery percentages (Tables 1 and 2). The standard addition method (three levels of addition, each in triplicate) was performed on the olive oil samples dissolved in Bz/EtOH (1:2) + 0.1 M H_2SO_4 using a carbon fiber disk UME as the working electrode (Table 1). For comparison, we also applied the same methodology after the extraction steps, where 10 mL of ACN + 0.1 M TBAHFP solutions containing extracted synthetic antioxidants were analysed with a Pt band UME as the working electrode, taking into account its very good sensitivity (Table 2) (see below). Very good linear relationships were obtained between $I_{p,n}$ versus $c^*_{A_{ox}}$ in all cases. Characteristic values for the linear regressions and recovery percentages are shown in Tables 1 and 2.

Recovery percentages in the range between 88% and 118% were obtained. The determined recovery percentages for the four synthetic antioxidants studied by the two methodologies were very similar. These results indicate that the extraction procedure was very efficient. The reproducibility was calculated from recoveries obtained by measuring three levels of addition (each in triplicate) by using fresh electrodes and solutions for each antioxidant. Percentage relative standard deviations (%RSD) in the range 1.5–4.1% and 1.5–6.6% were obtained for ACN + 0.1 M TBAHFP and Bz/EtOH (1:2) + 0.1 M H_2SO_4, respectively, as shown in Tables 1 and 2. The repeatability assays were performed from recoveries obtained on PG by measuring three consecutive identical levels of addition (each in triplicate, see samples 10–12 of Tables 1 and 2) on the same UME and starting PG solution. %RSD was about 1% in both reaction media (Tables 1 and 2).

Table 1
Recovery percentages obtained by the standard addition method from olive oil samples spiked with synthetic antioxidants by using SWV at a carbon disk UME (= 11 μm). Reaction medium: Bz/EtOH (1:2) + 0.1 M H_2SO_4. Reference electrode: SCE. $\Delta E_{sw} = 0.050$ V, $\Delta E_s = 0.005$ V and $f = 25$ Hz.

Sample	Synthetic antioxidant	10^{12} Slope[a]/A ppm^{-1}	10^{11} Intercept[b]/A	r^c	Spiked $c^*_{A_{ox}}$/ppm	Calculated $c^*_{A_{ox}}$/ppm	Recovery %	% RSD[d]
1	TBHQ	5.2 ± 0.5	4.3 ± 0.2	0.9905	80	90.4 ± 0.8	113	1.5
2		6.6 ± 0.6	5.6 ± 0.1	0.9990	100	115 ± 2	115	
3		7.1 ± 0.4	8.9 ± 0.1	0.9915	120	139 ± 1	116	
4	BHA	8.1 ± 0.4	9.1 ± 0.1	0.9998	50	49.0 ± 0.9	98	4.6
5		8.7 ± 0.3	4.9 ± 0.1	0.9816	70	62.3 ± 0.7	89	
6		9.1 ± 0.2	7.6 ± 0.1	0.9843	100	92 ± 1	92	
7	BHT	6.2 ± 0.2	4.7 ± 0.1	0.9980	100	118 ± 2	118	6.6
8		7.3 ± 0.4	0.10 ± 0.01	0.9823	140	158 ± 2	113	
9		8.1 ± 0.2	0.15 ± 0.01	0.9810	200	210 ± 3	105	
10	PG	5.8 ± 0.3	2.0 ± 0.1	0.9901	40	36.4 ± 0.5	91	2.5
11		6.1 ± 0.2	3.0 ± 0.1	0.9801	60	52.8 ± 0.8	88	
12		7.20 ± 0.07	15.2 ± 0.1	0.9995	106	99 ± 1	93	
13		6.80 ± 0.09	14.3 ± 0.2	0.9896	106	98 ± 1	92	
14		7.80 ± 0.05	16.7 ± 0.1	0.9932	106	100 ± 1	94	

a, b and c are the slope, the intercept and the linear correlation coefficient, respectively, of the $I_{p,n}$ (A) versus $c^*_{A_{ox}}$ (ppm) curve. d is the percentage relative standard deviation of the recovery obtained from three different spiked $c^*_{A_{ox}}$.

Table 2
Recovery percentages obtained by the standard addition method from olive oil samples spiked with synthetic antioxidants after extraction steps with ACN by using SWV at a Pt band UME. Reaction medium: ACN + 0.1 M TBAHFP. Reference electrode: SCE. ΔE_{sw} = 0.050 V, ΔE_s = 0.005 V and f = 25 Hz.

Sample	Synthetic antioxidant	10^8 Slope[a]/A ppm^{-1}	10^7 Intercept[b]/A	r[c]	Spiked $c^*_{A_{ox}}$/ppm	Calculated $c^*_{A_{ox}}$/ppm	Recovery %	RSD %[d]
1	TBHQ	1.31 ± 0.03	1.4 ± 0.1	0.9906	80	88.8 ± 0.4	111	1.5
2		1.49 ± 0.03	2.0 ± 0.2	0.9995	100	113.8 ± 0.4	114	
3		1.55 ± 0.02	2.5 ± 0.2	0.9895	120	134.4 ± 0.7	112	
4	BHA	1.53 ± 0.02	0.9 ± 0.1	0.9998	50	48.0 ± 0.1	969	3.1
5		1.61 ± 0.02	1.2 ± 0.2	0.9806	70	64.4 ± 0.4	290	
6		1.72 ± 0.03	1.8 ± 0.2	0.9815	100	90.0 ± 0.3		
7	BHT	0.82 ± 0.02	1.1 ± 0.1	0.9991	100	117.0 ± 0.7	117	4.1
8		1.12 ± 0.03	2.1 ± 0.1	0.9841	140	156 ± 1	111	
9		1.05 ± 0.01	2.7 ± 0.1	0.9850	200	218 ± 2	109	
10	PG	0.71 ± 0.01	0.39 ± 0.01	0.9901	40	46.0 ± 0.4	115	4.1
11		0.52 ± 0.01	0.42 ± 0.01	0.9804	60	67.2 ± 0.6	112	
12		0.25 ± 0.02	0.24 ± 0.01	0.9902	106	113.6 ± 0.5	107	
13		0.29 ± 0.02	0.28 ± 0.01	0.9915	106	114.8 ± 0.4112.4 ± 0.7	108	
14		0.21 ± 0.03	0.20 ± 0.02	0.9899	106		106	

a, b and c are the slope, the intercept and the linear correlation coefficient, respectively, of the $I_{p,n}$ (A) versus $c^*_{A_{ox}}$ (ppm) curve. d is the percentage relative standard deviation of the recovery obtained from three different spiked $c^*_{A_{ox}}$.

3.4. Qualitative analysis of binary and ternary antioxidant mixtures spiked to the olive oil matrix

We spiked olive oil samples with the same binary and ternary synthetic antioxidant mixtures described in Section 3.1. Extracts were dissolved in ACN + 0.1 M TBAHFP after extraction steps with ACN as indicated in Section 2.1. SW voltammograms recorded in the absence and in the presence of different aliquots of TBAOH allowed us to differentiate qualitatively the antioxidant mixture present in the oil sample. We describe the behaviour found for two of the mixtures studied and a summary for the other mixtures.

SW voltammograms obtained from the extract in the absence (line 1) and in the presence (lines 2 and 3) of different aliquots of the base when the olive oil matrix had been previously spiked with a mixture of 2.37×10^{-4} M (42.7 ppm) BHA + 1.88×10^{-4} M (40 ppm) PG are shown in Fig. 4A. Two SW voltammetric peaks were observed in the neutral medium at $E_{p,n} \cong 1.00$ V and 1.25 V, which can be assigned to BHA and PG, respectively. As the base was added to the reaction medium the peak current of both peaks decreased and new peaks appeared at lesser anodic potentials than the original voltammetric peaks. As can be observed, the PG voltammetric signal decreased faster than that of BHA. The diminution in PG signal could be associated with the increase of peak current at $E_{p,n} \cong 0.18$ V during the base successive additions, which can be assigned to the electrooxidation of one of three possible PG phenoxide ions. It appears anodically shifted about 0.09 V with respect to that found en pure solutions (Section 3.1), which can be explained considering a matrix effect. In addition, when the base concentration increased, the PG original peak showed a new diminution and two new characteristic peaks at $E_{p,n} \cong 0.42$ and 0.75 V were well defined, which can be assigned to the BHA phenoxide ion.

SW voltammograms performed on the extract obtained after extraction steps when the olive oil sample had been previously spiked with BHA + BHT + TBHQ mixture in the absence (line 1) and in the presence (lines 2 and 3) of the base are shown in Fig. 4B. Two main voltammetric peaks appeared at $E_{p,n} \cong 1.00$ V and 1.33 V in the neutral reaction medium, which can be assigned to BHA + TBHQ and BHT, respectively, by considering that BHA and TBHQ signals were previously found overlapping. In addition, three small peaks were observed a $E_{p,n} \cong -0.60$, 0.15, and 0.47 V (Fig. 4B, line 1) probably due to small amounts of oxidised antioxidants, mainly TBHQ and BHA. At a given base concentration, peaks at about $E_{p,n} \cong 0.16$, 0.42, and 0.75 V developed, which can be associated with the BHA phenoxide ion, and the peak current at $E_{p,n} \cong -0.60$ V increased. The latter peak can be assigned to the presence of TBHQ phenoxide ion in the reaction medium (Fig. 4B, line 2), which is better defined as the base concentration increases (Fig. 4B, line 3). The characteristic peak of BHT (−0.40 V) is not observed in this experimental set because of the high base concentration. As it was explained elsewhere (Richards, Whitson, Evans, 1975), the BHT phenoxide ion system appearance is very sensitive to base concentration, i.e., voltammetric peaks are only observed when almost equivalent amounts of antioxidant and base are present. However, the presence of the peak at 1.30 V in the neutral medium can only be assigned to BHT in the mixture.

We found a similar behaviour to that previously described for the other mixtures studied (results not shown). Therefore, the BHA + BHT mixture showed two peaks in absence of the base, with $E_{p,n} \cong 1.00$ V and 1.30 V. Other two peaks at $E_{p,n} \cong 0.40$ V (BHT) and 0.74 V (BHA) were well defined in the presence of the base. The BHA + TBHQ mixture showed only one peak centered at $E_{p,n} \cong 1.00$ V in the neutral medium. The main characteristic peaks for BHA and TBHQ phenoxide ions were found in the presence of the base, with $E_{p,n} \cong 0.74$ V and −0.60 V, respectively. The BHT + PG mixture showed only one peak at $E_{p,n} \cong 1.30$ V in the neutral medium and the PG characteristic voltammetric signals in the potential range from 0.00 to about 0.60 V were also observed. Two new peaks at about $E_{p,n} \cong 0.06$ V and 0.40 V were observed in the basic medium, corresponding to PG and BHT phenoxide ions, respectively.

The BHA + BHT + PG mixture showed two peaks at about $E_{p,n} \cong 1.00$ V (BHA) and 1.30 V (BHT, PG), and the PG characteristic voltammetric signals in the potential range from 0.00 to about −0.80 V. Moreover, peaks centered at about $E_{p,n} \cong 0.74$, 0.40, and 0.20 V were found in the basic medium. However, the BHT phenoxide ion oxidation was not observed in this mixture.

As it was previously shown, it is possible to identify the antioxidant components of mixtures in edible oil samples by comparison of voltammetric responses in a neutral solution and after successive additions of base without using chemmometric approaches to bypass problems of overlapped peaks.

4. Conclusions

Firstly, we could accomplish a qualitative determination of permitted synthetic antioxidant mixtures in edible oils based on the different acid–base properties of antioxidants in acetonitrile solutions. Therefore, the binary mixtures BHA + BHT, BHA + PG, BHA + TBHQ, and BHT + PG as well as the ternary mixtures BHA + TBHQ + BHT and BHA + BHT + PG could be resolved by

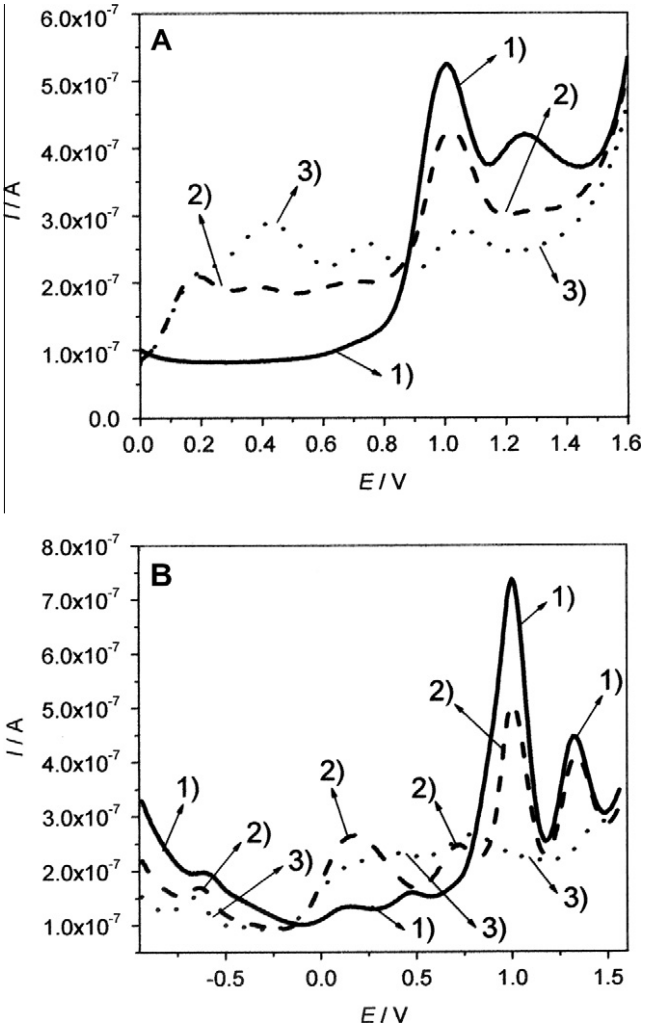

Fig. 4. (A) SW voltammograms obtained in ACN + 0.1 M TBAHFP after extraction steps with ACN when the olive oil matrix had been previously spiked with 2.37×10^{-4} M (42.7 ppm) BHA + 1.88×10^{-4} M (40 ppm) PG in the absence (solid line, 1) and in the presence of TBAOH (dash line, 2 and dot line, 3). $c^{*}_{TBAOH} = 3.43 \times 10^{-4}$ M and 6.85×10^{-4} M for lines 2 and 3, respectively. (B) SW voltammograms obtained in ACN + 0.1 M TBAHFP after extraction steps with ACN when the olive oil matrix had been previously spiked with 5.54×10^{-5} M (10 ppm) BHA + 4.52×10^{-5} M (10 ppm) BHT + 1.2×10^{-4} M (20 ppm) TBHQ in the absence (solid line, 1) and in the presence (dash line, 2 and dot line, 3) of base. $c^{*}_{TBAOH} = 2.76 \times 10^{-4}$ M (line 2) and 7.18×10^{-4} M (line 3). Other experimental conditions are the same as Fig. 1.

comparison of characteristic voltammetric peaks obtained in neutral medium and after the successive additions of $(C_4H_9)_4NOH$.

Secondly, we transferred these results to the study of olive oil samples with no synthetic antioxidant added (according to the manufacturer's label). Olive oil samples were spiked independently with a given amount of TBHQ, BHA, BHT, and PG dissolved in Bz/EtOH (1:2) + 0.1 M H_2SO_4. The standard addition method was employed to obtain the recovery percentages both in the sulphuric acid medium and after extraction steps with acetonitrile. Recovery percentages in the range from 88% to 118% were determined. Moreover, the recovery percentages obtained for each antioxidant in both reaction media were very similar showing clearly that the extraction procedure was very efficient. It is concluded that the respective systems exhibited a good percentage recovery, reproducibility, repeatability and linear range for the quantification of synthetic antioxidants. In addition, olive oil samples spiked with the different binary and ternary permitted synthetic antioxidant mixtures were studied in ACN + 0.1 M TBAHFP after extraction steps with ACN. SW voltammograms recorded in the absence and in the presence of different aliquots of TBAOH allowed differentiation of the antioxidants added.

Acknowledgements

S.N. Robledo thank the Doctoral fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. We wish to thank to the Secretaría de Ciencia y Técnica (SECyT) from the Universidad Nacional de Río Cuarto and CONICET for financial supports.

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