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Virgin olive oil *ortho*-phenols—electroanalytical quantification

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ABSTRACT

An electroanalytical methodology was developed for the determination of the total *ortho*-phenol content of virgin olive oil (VOO) with high sensitivity and reproducibility. The VOO *ortho*-phenol content depends on its freshness and is normally expressed as HT equivalent. Screen-printed electrodes were used with cyclic voltammetry to investigate the oxidation of catechol, phenol, hydroxytyrosol (HT), tyrosol, caffeic acid and ferulic acid. The oxidation of *ortho*-phenols and *mono*-phenols occurs following different mechanisms, and at different potentials. Using screen-printed electrodes and square wave voltammetry, an HT detection limit of 0.40 μM, was obtained. The electroanalytical methodology developed was applied to the determination of *ortho*-phenol content in fresh and old VOO. The HT equivalent determined for a two-year-old VOO sample was 3 mg/kg, for one-year-old samples was 6–7 mg/kg, and for a fresh VOO sample 30 mg/kg, recoveries in the range of 78–93% of HT standard being obtained. The effect of VOO matrix components on the HT standard response was investigated.

1. Introduction

The beneficial effects of virgin olive oil (VOO) can be attributed not only to the high relationship between unsaturated and saturated fatty acids of olive oil, but also to the antioxidant properties of its phenolic compounds [1,2]. Phenols make up a part of the so-called "polar fraction" of VOO, which is usually obtained by extraction with methanol/water mixtures [2,3]. The olive pulp contains these compounds, which are hydrophilic, but they are also found in the oil [3]. This class of phenols includes numerous components, simple phenolic compounds, such as vanillic, gallic, coumaric and caffeic acids, tyrosol and hydroxytyrosol and more complex compounds such as the secoiridoids oleuropein and ligstroside, and the lignans acetoxypinoresinol and pinoresinol, Scheme 1 [4,5].

VOO is produced using only the cold-pressing method, without further treatment other than washing, filtration, decantation, or centrifugation, and is composed of a triglyceride fraction (up to 90–99% of the olive fruit) and a non-glycerol or unsaponifiable fraction (0.4–5% of the olive fruit) which contains phenolic compounds [2–6]. The phenolic fraction contains at least 36 structurally distinct phenolic compounds, but the widespread phenols of VOO are the secoiridoids, that are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure [2–5,7]. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside, form the majority of the phenolic fraction [5,7,8]. However, the most abundant secoiridoids of VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol and an isomer of the oleuropein aglycone [5].

Variations in the phenolic concentration between different VOO are due to numerous factors including: the variety of the olive fruit, the region in which the olive fruit is grown, the agricultural techniques used to cultivate the olive fruit, the maturity of the olive fruit at harvest, and the olive oil extraction, processing, storage methods and time since harvest [9].

Various studies *in vivo* and *in vitro* demonstrated that olive oil phenolic compounds have positive effects on certain physiological parameters, such as plasma lipoproteins, oxidative damage, inflammatory markers, platelet and cellular function, antimicrobial activity and bone health [1–5].

Phenolic compounds can act as antioxidants in various ways. In oxidative systems using transition metals such as Cu and Fe, they can chelate metal ions, which can prevent their involvement in Fenton reactions that can generate high concentrations of hydroxyl radicals [10,11]. However, the most important antioxidant activity is related to the free radical-scavenging ability, by breaking the chain of reactions triggered by free radicals [2,3,7–9].

Numerous studies on the phenols have shown that the degree of antioxidant activity is correlated with the number of hydroxyl groups [9,12–15]. In particular, *ortho*-phenolic substitution, as in hydroxytyrosol, gives high antioxidant ability, while a single hydroxyl substitution, as in tyrosol, does not confer any activity,

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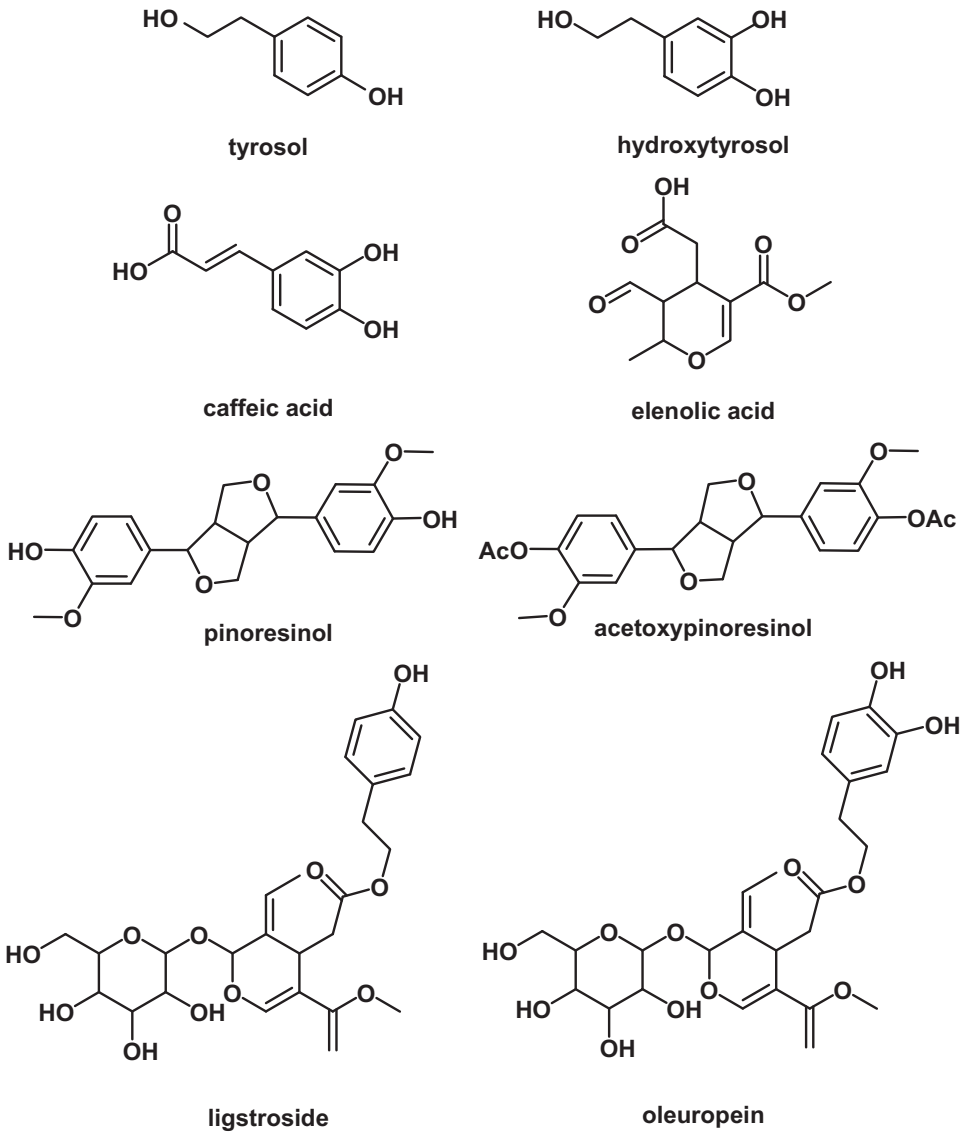

Scheme 1. Chemical structures of phenols present in virgin olive oil (VOO).

since tyrosol does not protect LDL from chemically induced oxidation [8,15].

As a consequence of the antioxidant activity and health benefits there is increasing interest in the determination of the concentration of VOO *ortho*-phenols. The current analytical procedure involves three basic steps: extraction from the oil sample, analytical separation, and quantification [1,5,7,8]. A large number of analytical determinations, based on spectrophotometric methods, after analytical separation, by gas chromatography, high-performance liquid chromatography, and capillary electrophoresis, have already been developed and described [5,7–9,15,16]. However, these methods are expensive, need pre-treatment and require skilled operators.

Voltammetric methods, due to their high sensitivity, have been successfully used for the electrochemical investigation of biological active substituted phenols, providing valuable results [17–19]. The analytical determination of the mostly widespread phenols in aqueous solutions and in complex aqueous food samples, such as teas, wines, beers and others, has been previously investigated using electrochemical techniques at different electrode materials: glassy carbon, carbon paste, boron doped diamond, etc. [17,20–25].

Oxidation of *mono*-phenols is an irreversible process, occurring in one step at a relatively high potential, whereas *ortho*-phenol oxidation occurs at a low potential in a two-electron–proton reversible mechanism [26].

The present study is concerned with the development of a selective electroanalytical method for the determination of VOO total *ortho*-phenol content using square wave voltammetry at screen printed electrodes. This will provide an easy method for VOO total *ortho*-phenol quantification, inexpensive, portable and with the possibility of using a miniaturized device.

2. Experimental

2.1. Materials and reagents

Phenol (Ph), catechol (CT), tyrosol (T), hydroxytyrosol (HT), ferulic acid (FA) and caffeic acid (CA) from Sigma were used without further purification. Stock solutions of 10 mM were prepared in ultra-pure water, ethanol and methanol (1:1:1) and were stored at $+4$ °C.

The supporting electrolyte, 0.1 M phosphate buffer pH=7.0, was chosen due to the higher oxidation peak potentials of phenol derivatives [26]. The supporting electrolyte solution also contained 0.05 M KCl, since a silver pseudo-reference electrode was used. All solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity $<0.1\ \mu S\ cm^{-1}$).

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co. Inc., Woburn, USA). All experiments were done at room temperature ($25\pm1\ ^{\circ}C$).

The virgin olive oils (VOO) were purchased in local markets (Mohammedia, Morocco and Coimbra, Portugal). These samples were characterized by a free fatty acid content of less than 0.8 g per 100 g VOO and a peroxide value <20 milliequivalents of active oxygen/kg oil.

2.2. Voltammetric parameters and electrochemical cell

Voltammetric experiments were carried out using a PalmSens portable electrochemical analyzer running with the PalmSensePC 2.6 software, Palmsens BV, Houten, Netherlands.

Cyclic voltammetry (CV) used a scan rate 30 or 50 mV s^{-1}. For square wave (SW) voltammetry, the parameters were: pulse 25 mV, frequency 12 Hz and potential increment 2 mV, corresponding to an effective scan rate of 50 mV s^{-1}. Measurements were carried out using disc-shaped screen-printed electrodes (SPEs) ($d=3$ mm) in a 10 mL one-compartment electrochemical cell.

The SPEs consisted of three electrodes: two carbon electrodes as working and counter electrodes, and a silver pseudo-reference electrode, and were a gift from University of Rome "Tor Vergata". The SPEs were produced using a 245 DEK (Weymouth, England) screen-printing machine. Graphite-based ink (Electrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrodes. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). An insulating ink was finally used to define the working electrode surface ($\Phi=3$). A curing period of 10 min at 70 °C was applied [24]. Prior to use, several procedures were tested in order to activate the electrode surface.

2.3. Screen printed electrode activation

The SPEs were washed with ethanol and ultra-pure water, and three different electrochemical procedures were investigated for surface activation using 0.1 M phosphate buffer pH=7.0. After activation, cyclic voltammograms were recorded in 50 μM catechol in 0.1 M phosphate buffer pH=7.0, Fig. 1. The activation procedure and the results obtained were:

- *procedure* 1—a positive potential of +1.7 V was applied for 120 s, $\Delta E_p=+0.63$ V was found, Fig. 1;
- *procedure* 2—a negative potential of −1.0 V was applied for 120 s, $\Delta E_p=+0.34$ V was found, Fig. 1 and
- *procedure* 3—a negative potential of −0.8 V was applied for 120 s, and afterwards the electrode was cycled, between the potentials $E_1=-1.0$ V and $E_2=+1.7$ V, until a stable signal was detected (~10–15 cycles at scan rate 50 mV s^{-1}), $\Delta E_p=+0.048$ V was found, Fig. 1.

Considering the peak currents of 0.63, 0.83 and 1.46 μA, for *procedures* 1, 2 and 3, respectively, the results showed that the best sensitivity was achieved following *procedure* 3, and this procedure was chosen for all electrochemical experiments.

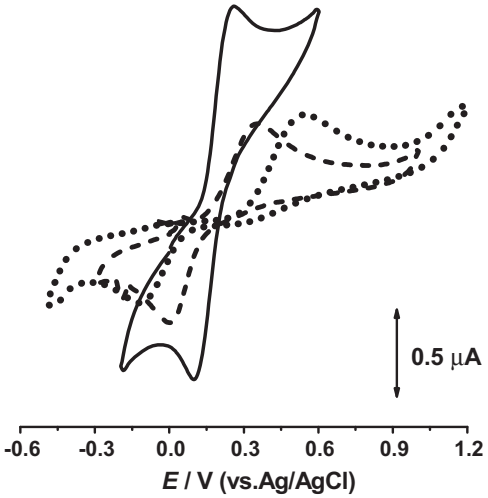

Fig. 1. CVs in 50 μM catechol in 0.1 M pH=7.0 phosphate buffer using different activation procedures: (●●●) *procedure* 1, (■■■) *procedure* 2 and (▬▬) *procedure* 3.

2.4. Extraction of the phenolic fraction from VOO samples

Analytical procedures applied to the determination of organic compounds in natural solid/liquid samples need the use of a convenient extraction procedure that will not influence the accuracy of the results, and the total analysis time and cost can also be affected by sample throughput. Several efficient extraction techniques have been developed and are commonly used for isolation of phenolic compounds from oil matrices [5].

The phenolic fraction was extracted from 25 g VOO samples and was dissolved in 25 mL hexane, and the polar compounds were extracted with a mixture of methanol/water (3:2, v/v, 3×15 mL), at room temperature. The extraction was completed by addition of ultra-pure water, the final volume being 50 mL.

3. Results and discussion

The objective and innovation of this work is to provide an olive oil freshness indicator, so the selectivity of voltammetric methods for the electrochemical detection of *ortho*-phenols with respect to *mono*-phenols, at SPEs, was studied in detail. Following this, an SW voltammetry electroanalytical procedure for analysis of VOO extracts, using HT as external standard, was developed.

3.1. Voltammetric behaviour of ortho- and mono-phenols

Cyclic voltammograms of the oxidation of the *ortho*- and *mono*-phenols studied are shown in Fig. 2.

HT and CA have a catechol moiety whereas T and FA have only one hydroxyl group in a phenol moiety. The oxidation of catechol and phenol occur each with one peak and at different potentials, Fig. 2A.

CVs recorded in 50 μM catechol, HT and CA solutions, in 0.1 M phosphate buffer pH=7.0, from +0.00 V to +0.60 V, using newly activated SPEs, showed one anodic peak, at E_{pa} ~+0.11 V, and after reversing the scan direction, from +0.60 V to −0.20 V, a cathodic peak, at E_{pc} ~0.06 V, appeared, Fig. 2, showing the reversibility of the oxidation reactions.

The cathodic peak corresponds to the reversible reduction of the catechol moiety oxidation product, *ortho*-quinone, to the *ortho*-phenol moiety. The difference between the anodic and the cathodic peak potentials $|E_{pa}-E_{pc}|\sim30$ mV agrees with a two-electron reversible reaction [27]. Moreover, the ratios of the anodic, I_{pa},

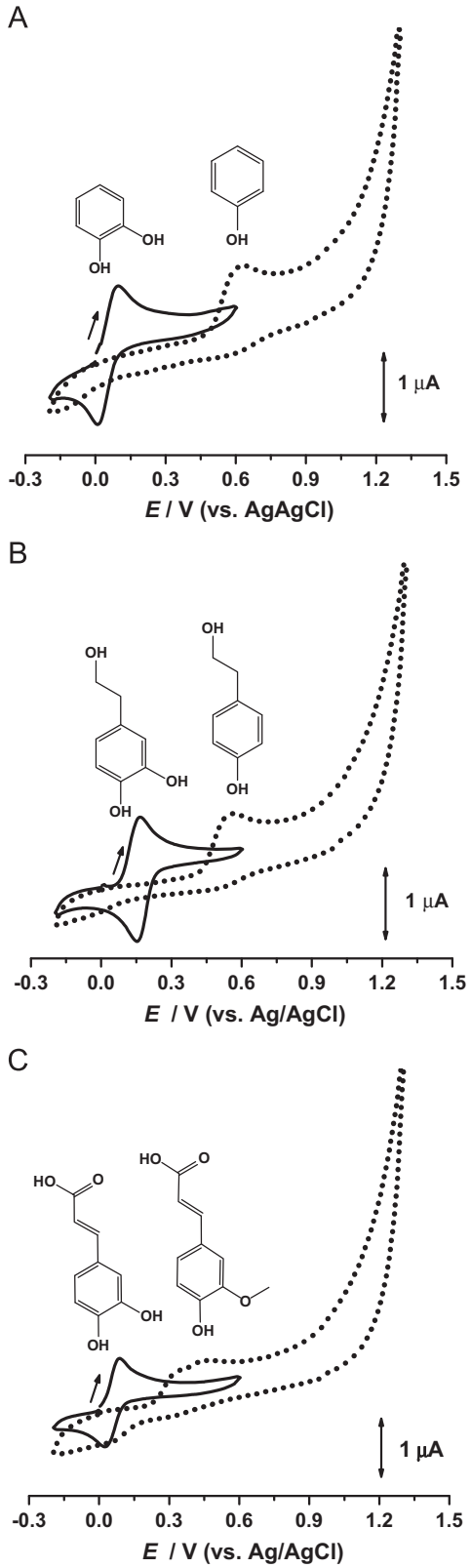

Fig. 2. CVs in 0.1 M phosphate buffer pH=7.0 using SPEs in 50 μM: (A) (▬) catechol and (●●●) phenol, (B) (▬) HT and (●●●) T, and (C) (▬) CA and (●●●) FA; ν=30 mV s^{-1}.

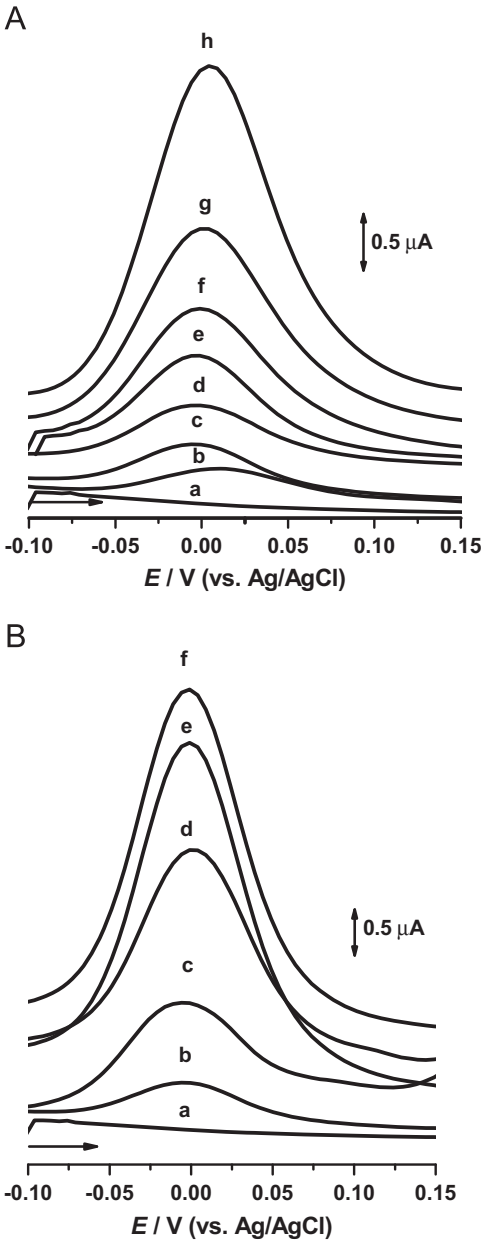

Fig. 3. SW voltammograms in 0.1 M phosphate buffer pH=7.0: **(A) a**- 0, **b**- 1, **c**- 2, **d**- 4, **e**- 10, **f**- 20, **g**- 30, and **h**- 50 μM HT using a new SPE for each concentration; (B) **a**- 0, **b**- 2, **c**- 4, **d**- 8, **e**- 16 and **f**- 32 μM HT using always the same SPE.

and cathodic, I_{pc}, peak currents of catechol, HT and CA are all close to one (1.0 ± 0.1).

CVs recorded in 50 μM phenol, T and FA solutions, in 0.1 M phosphate buffer pH=7.0, from +0.00 V to +1.2 V, using newly activated SPEs, showed one anodic peak, at E_{pa} ~ +0.6 V, and after reversing the scan direction, from +0.60 V to −0.2 V, no cathodic peak appeared, Fig. 2, showing the irreversibility of these oxidation reactions.

Thus, at newly activated SPEs, the oxidation of *mono*-phenols occurs at a much higher potential than that of the *ortho*-phenols, and they follow different mechanisms, in agreement with previously published results [26].

3.2. Electroanalytical determination of HT

The advantages of SW voltammetry are greater speed of analysis, lower consumption of the electroactive species in relation with differential pulse voltammetry, and reduced problems with poisoning of the electrode surface [27], which represents a main limitation in the direct electrochemical detection of polyphenols in real samples.

In order to develop an electroanalytical detection method of total *ortho*-phenols in natural VOO samples, HT was chosen as standard, and its quantification using SPEs was investigated by SW voltammetry.

The effect of interference from other molecules, present in the natural VOO matrix, on the electrochemical behaviour of HT was investigated in a *mono*-phenolic synthetic solution.

3.2.1. Calibration plots of HT standard

SW voltammograms were recorded at a newly-activated SPE for standard additions of HT corresponding to bulk concentrations between 2 and 50 μM, Figs. 3 and 4A.

The HT detection limit (LOD) was determined from the equation $LOD = 3 \times SD \times (\text{sensitivity})^{-1}$, where SD is the standard deviation of the response, and the sensitivity is the slope of the calibration plot. The quantification limit (LOQ), the lowest concentration that can be quantified with acceptable precision and accuracy, is given by $LOQ = 10 \times SD \times (\text{sensitivity})^{-1}$.

The electroanalytical determination of HT carried out by SW voltammetry, measuring the total oxidation peak current of HT, gave a LOD of 0.40 μM and a LOQ of 1.35 μM. Taking into account the procedure of *ortho*-phenol analysis (25 g olive sample/50 mL extract, dilution factor 1:10, 1 mL of the extract in 9 mL of electrolyte cell solution), Sections 2.3 and 3.3.1, this LOD corresponds to 1.25 mg/kg, which is much lower than the value that is expected in a natural sample, e.g. VOO, where the concentration of *ortho*-phenols, expressed as HT, is higher, in the range of 10–100 mg/kg (2.93–29.3 μM) [7,23].

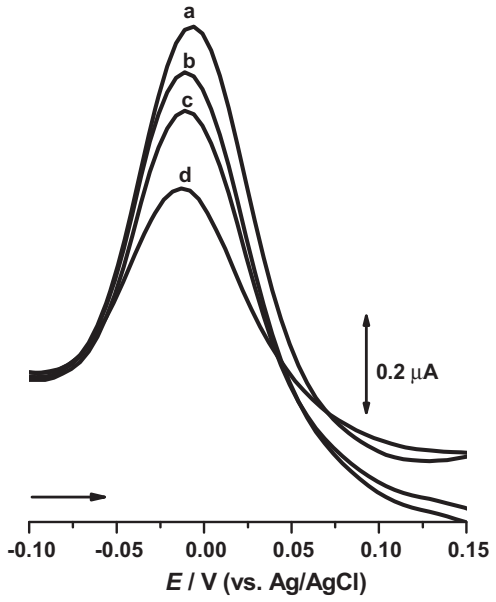

Fig. 4. SW voltammograms in 10 μM HT in 0.1 M phosphate buffer pH = 7.0 using SPEs in the presence of mono-phenols: **a**- 0, **b**- 10 μM phenol + 10 μM tyrosol, **c**- 30 μM phenol + 30 μM tyrosol and **d**- 50 μM phenol + 50 μM tyrosol.

Table 1
Variation of 10 μM HT standard peak potential and current with different total concentrations of phenol and tyrosol (1:1).

***mono*-phenols (1:1) (μM)**	E_p **(V)**	I_p **(μA)**	**HT (%)**
0	−0.006	0.83	100
20	−0.009	0.78	94
60	−0.010	0.67	81
100	−0.011	0.47	57

Each measurement was always done using a newly-activated SPE, a process that gives rise to small changes in the electrode surface area, which can in turn cause small variations in the currents measured, and this should be the main reason for the difference in the results obtained using the procedure described. The data extracted from the five calibration curves, demonstrates, by the values of $R^2 = 0.99$, a wider linear range following the equation $I_{pa} = 0.079 \pm 0.002\ c + 0.041 \pm 0.008$ μA, where c is expressed in μM. The relative standard deviation (R.S.D.), calculated from five calibration curves, was less than 12%.

The effect of HT adsorption on the electrode surface was then evaluated. Successive SW voltammograms were recorded in HT solutions of increasing concentration always using the same SPE, Fig. 3B. The calibration curve showed saturation effects above 8 μM HT, because the HT oxidation/reduction products adsorb at the electrode surface, and cumulative adsorption of redox species causes an increase in current with a loss of reproducibility and sensitivity. Adsorption may be reduced by using a modified electrode surface and improving the reproducibility and sensitivity [28].

3.2.2. Interference from mono-phenols

The interference from different *mono*-phenols on HT determination was investigated. SW voltammograms in 0.1 M phosphate buffer pH = 7.0, using a newly activated SPE, in 10 μM HT in the presence of different concentrations of phenol and tyrosol (T) showed no variation in the HT oxidation peak potential of $E_p \sim 0.0$ V, but the peak current decreased linearly with increasing concentration of *mono*-phenols, Table 1. This effect is explained

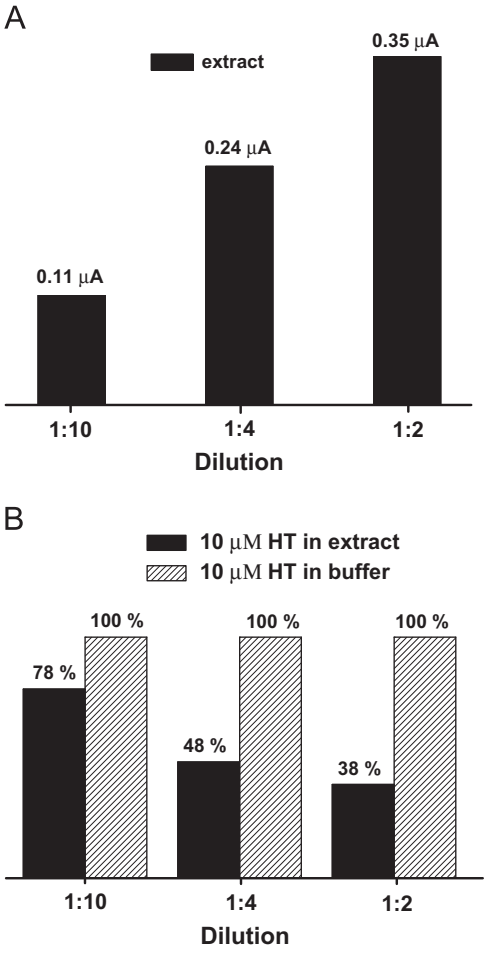

Fig. 5. (A) Peak currents from SWVs of extract as function of dilution factor (Df) and **(B)** Peak currents from SWVs of standard in extract vs. standard in buffer.

considering that phenol and tyrosol both adsorb on the electrode surface forming a non-compact monolayer. Further oxidation of HT molecules, diffusing from the bulk solution towards the electrode surface, is more difficult because the electrode surface area is decreased by the layer of adsorbed *mono*-phenols.

3.3. Electroanalytical determination of ortho-phenols in natural samples

The electroanalytical method developed was used for determination of *ortho*-phenols in natural VOO sample extracts using HT as the external standard and the matrix effect was also evaluated.

3.3.1. Matrix effect

Different dilutions of the VOO extracts, prepared according to Section 2.3, Fig. 5, were investigated in order to minimize the degree of electrode fouling due to the adsorption of the matrix VOO extract components while maintaining the electroanalytical conditions for good reproducible detection of *ortho*-phenols.

SW voltammograms recorded using new, activated SPEs in 0.1 M phosphate buffer pH = 7.0 using VOO extracts (dilution

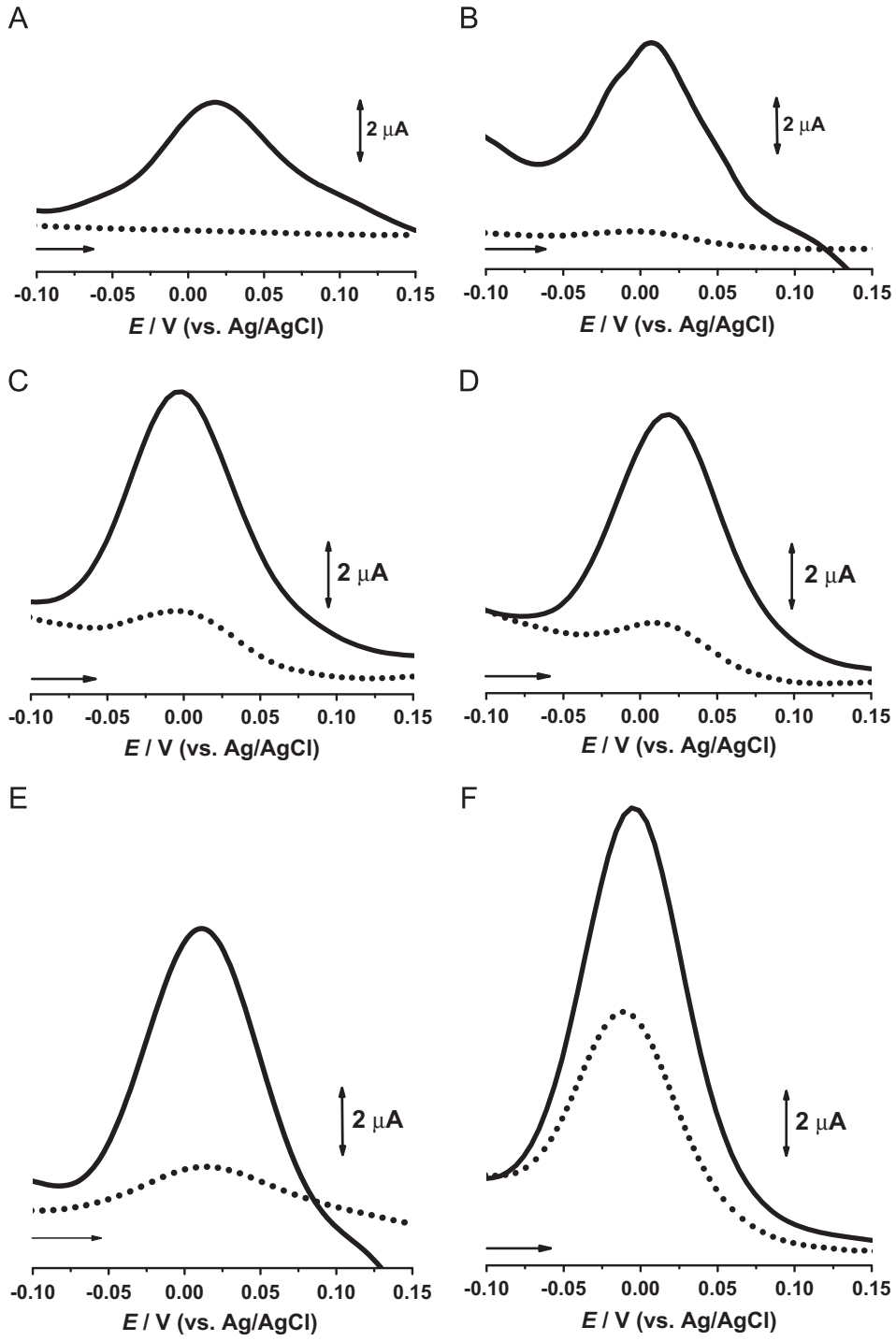

Fig. 6. SW voltammograms in 0.1 M phosphate buffer pH = 7.0 using SPE in different VOO samples: **(A)** ten-years-old, **(B)** two-years-old, **(C)**, **(D)** and **(E)** one-year-old and **(F)** new; (●●●) extract and (▬) extract +10 μM HT.

factors 1:10 to 1:2) showed that the current response did not change proportionally with the dilution factor, Fig. 5A. When 10 μM HT was added to the VOO extract samples, particularly for a low dilution factor (1:2), a decrease of the HT standard current was observed. The decrease of HT electrochemical response shows that electrode passivation by matrix component adsorption is strongly related to the dilution factor.

Although the oxidation peak current of HT in VOO extract samples increased with decrease in VOO extract concentration, Fig. 5B, the current corresponding to the *ortho*-phenols in the VOO extract samples obtained for the highest dilution (1:10) is close to the detection limit, which is in the range of 1.25 mg/kg. Further dilution of the extract would lead to a concentration of the *ortho*-phenols below the LOD. Thus, optimization of the analysis procedures requires 1:10 dilution of VOO extract in order to avoid electrode fouling and the interference of electroactive VOO extract components.

3.3.2. Olive oil analysis

The total *ortho*-phenol content of six VOO extracts was evaluated by SW voltammetry and calculated as HT equivalent: two extracts were prepared using a ten-year-old (S1) and a two-year-old (S2) samples from Portugal; three extracts were prepared using different one-year-old VOO, two samples (S3 and S4) from Morocco and one sample (S5) from Portugal, and one extract was prepared from a new VOO sample (S6) from Morocco. A dilution factor of 1:10 was used and 10 μM HT standard was then added.

The ten-year-old sample (S1) gave no electrochemical response, Fig. 6A. On the other hand, the response obtained for the two- and one-year-old VOO extracts (S2, S3, S4 and S5), Fig. 6B–E, showed similar behaviour. However, a large increase of current occurred with the new VOO extract (S6), Fig. 6F. The results in Table 3 are the average of three determinations of *ortho*-phenol content. The R.S.D. of one-year-old and new VOO samples varied from 3% to 15%, the highest error corresponding to old VOO samples, which contain a very low amount of *ortho*-phenols. Moreover, due to the low *ortho*-phenol content and high concentration of degraded species, which increase the matrix effect, a very high error was obtained for a two-year-old sample.

It should be mentioned that after standard addition to the VOO extract solutions a loss of 25–30% of HT standard signal for *ortho*-phenols was observed, in comparison to the signal of HT as standard only. Although sample dilution can improve the HT response, there were some limitations related to LOD of *ortho*-phenols. Also, it should be mentioned that the concentration of all components of the phenolic fraction of VOO, other than *ortho*-phenols, are higher than the *ortho*-phenol concentration, and increase with time.

This data can be compared with the results obtained for HT standard in the presence of different concentrations of *mono*-phenols, phenol and tyrosol, Table 1, meaning that the concentration of VOO extract components which affects the electrochemical signal of HT standard is in the range of 40–50 μM as phenol or tyrosol equivalent.

The *ortho*-phenol concentrations in old VOOs obtained as HT equivalent were 3 mg/kg for two-year-old and 6–7 mg/kg for one-year-old samples, whereas for a ten-year-old VOO no *ortho*-phenol was detected. However, in new VOO the concentration of *ortho*-phenols was 30 mg/kg, Table 2. Nevertheless, the content of total *ortho*-phenol in the samples employed in this work was also determined using a colorimetric method [29]. This method is not yet recommended by the International Olive Oil Council but gives a rapid estimation of *ortho*-phenols. Indeed, an intense colour was observed with sample S6 (fresh oil) compared to the other samples (old oils) in agreement with the results of Table 2.

Table 2
VOO *ortho*-phenol concentration expressed as HT equivalent.

Olive oil	[*ortho*-phenols] (mg/kg)	R.S.D. (%) $n=3$
S1	–	–
S2	3.62 ± 0.91	25
S3	6.63 ± 0.74	11.2
S4	5.92 ± 0.88	15
S5	7.69 ± 0.71	9
S6	30.4 ± 1.00	3.3

Table 3
VOO matrix effect on HT standard.

VOO	A	B	C	Recovery (%) $(\frac{C-A}{B}) \times 100$
	ortho-phenols in extract (mg/kg)	Standard addition (mg/kg)	*ortho*-phenols found (mg/kg)	
Old—1	5.92	9.25	13.1	78
Old—2	5.92	30.8	34.5	93

The recovery using the HT standard electroanalytical method was investigated by spiking an old VOO extract at two concentration levels, 9.25 mg/kg and 30.8 mg/kg, Table 3. The HT standard was sufficiently recovered in the range of 78–93% with 1–3% of R.S.D., for two spiked concentration levels.

4. Conclusions

A rapid, efficient and sensitive electroanalytical method for the determination of total *ortho*-phenolic compounds in VOO extracts was developed using SW voltammetry and SPEs. The experimental conditions, including the electrode activation method, choice of dilution factor of VOO extract, as well as the matrix effect on the HT standard, were investigated. The oxidation of *ortho*- and *mono*-phenols occurs by different mechanisms, and at different potentials. A detection limit for HT, LOD = 0.40 μM corresponding to 1.25 mg/kg, was obtained. The effect of VOO matrix components on the electrochemical behaviour of HT was investigated in a *mono*-phenolic synthetic solution.

The *ortho*-phenol concentration determined as HT equivalent for two-years-old VOO extract was 3 mg/kg, for one-year-old VOO extracts was ~6–7 mg/kg, for a new VOO extract it was 30 mg/kg whereas for a ten-year-old VOO no *ortho*-phenol was detected, showing that the proposed procedure can be applied as an olive oil freshness indicator.

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