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Abstract

The quality of olive oil is essentially determined by the product free acidity and peroxide value, while the total phenol content is also important for a high antioxidant capacity. Generally, these parameters are measured with laboratory analysis, that are expensive and may require a few days. Thus, a cheap and easy technique usable by untrained personnel, "on-site" and producing results "in real time" during production is desirable, particularly as far as small olive oil mills and packaging centers are concerned. This paper describes a technique to determine peroxide value and total phenol content in olive oil, that is based on the measurement of optical density of an emulsion between a suitable chemical reagent and a small quantity of the oil of interest. The optical density is measured by illuminating the sample with a LED with peak wavelength of 569 nm for peroxide value and 835 nm for total phenol content. The experimental results show good correlation (R^2 = 0.883 and 0.895 for peroxide value and total phenol content, respectively) between data measured with the standard methodology and the technique of this work, implemented also in the form of a portable embedded system.

Keywords: olive oil, peroxide value, total phenol content, sensor, optical density.

1. Introduction

Olive oil is a vegetable lipid obtained by extraction process from olives (the fruits of *Olea europaea L.*, family Oleaceae) highly appreciated for its beneficial effects on human health, mainly due to a high content of oleic acid and phenolic compounds (Tulipani et al., 2012). Clinical studies provide evidence that regular olive oil consumption reduces the risk of coronary heart diseases (Keys et al., 1986), oxidative damage to DNA and RNA (Machowetz et al., 2007) and Alzheimer disease (Abuznait et al., 2013; Monti et al., 2011).

Olive oil quality is related to its chemical composition, oxidative stability and sensory characteristics. Quality parameters, such as free acidity, peroxide value, UV extinction coefficients, fruity attribute, other sensory characteristics and defects, are strongly dependent on olives' ripeness (Rotondi et al., 2004) and processing technology in the olive mills (Boselli et al., 2009). In addition, the peroxide value, defined as milliequivalent of active oxygen per kilogram of oil (meq O_2/kg oil) and qualifying the oil primary oxidation, is also related to storage conditions (oxygen, light exposure and temperature) after production. Another important quality parameter is the amount of phenolic compounds that contribute to the oil sensory taste producing a distinctive bitter and a pungent perception (Gutierrez-Rosales et al., 2003). Phenolic compounds found in olive oil are principally secoiridoids (oleuropein and ligstroside isomers) and their derivatives, such as tyrosol and hydroxytyrosol, that exhibit a strong antioxidant activity: they act as free radicals traps protecting from heart disease and displaying anticancer activity (Notarnicola et al., 2011; Zanoni, 2014). Phenolic compounds are also largely responsible for the shelf-life of the oil (Lerma-Garcia et al., 2009).

The European Commission regulation No. 2568/91 and subsequent amendments define manual titration methods to measure acidity and peroxide value in olive oil (EEC 2568, 1991), to be carried out in a laboratory environment by trained personnel. Instead, no official determination is currently established for the total phenol content, usually determined using spectrophotometry or high

performance liquid chromatography (HPLC), techniques requiring expensive instrumentation, a laboratory environment (IOC/T.20/Doc No 29, 2009; Tasioula-Margari and Okogeri, 2001) as well as preventive extraction of the polyphenols.

From the production point of view, the need to ship oil samples to a laboratory for analysis leads to high costs and long delays. Therefore, simple and fast techniques useable for on-site quality control are desirable, in particular for small oil mills and packaging centers. For this reason, innovative solutions have been proposed, such as: Near-InfraRed (NIR) spectroscopy (Armenta et al., 2007; Ozdemir and Ozturk, 2007) to estimate acidity and peroxide value; Time Domain Reflectometry (TDR) to determine water content (Ragni et al., 2012) and detect adulteration (Cataldo et al., 2012) in extra virgin olive oil; Rapid Fourier Transformed Infrared (FTIR) spectroscopy (Cerretani et al., 2010) and voltammetric sensors (Rodriguez-Mendez et al., 2008) to estimate total phenol content. However, all these techniques require expensive instrumentation and/or need frequent calibration for olives of different varieties, country of origin and harvest season.

As viable alternatives, amperometric and pH-metric techniques have been proposed to measure peroxide value (Kardash-Strochkova et al., 2001; Adhoum and Monser, 2008) and total phenol content (Capannesi et al., 2000), but these methods are still at research stage and have been validated only on small amounts of samples in laboratory environment. Moreover, some techniques use toxic compounds (such as chloroform) to increase oil solubility in reagents, unsuitable for use in normal working environment.

Recently, we have proposed a novel technique based on Electrical Impedance Spectroscopy to measure olive oil acidity that is fast (response time in about 30 seconds) and can be easily implemented in the form of a low-cost portable embedded system (Grossi et al., 2013).

To complete this work, we here present a simple and effective technique to measure peroxide value and total phenol content in olive oil that, as will be shown, is fast, accurate and can be implemented in the form of a low-cost embedded electronic system.

2. Materials and methods

2.1 Technique

The technique used in this work is based on the creation of an aqueous emulsion between the oil sample and a chemical reagent. The optical density (OD) of such an emulsion is determined by illuminating the sample with a LED and measuring the transmitted light through the sample with a photodiode. A large set of experimental results show a good correlation between the measured OD and the quality parameters determined by reference methods. The proposed technique is suitable to be implemented in the form of a portable instrument suitable for quick in-situ quality control, as will be discussed in sub-Section 3.3.

2.2 Experimental set-up

In order to validate the technique used in this work, measurements on olive oil samples have been initially carried out using an ad-hoc experimental set-up of bench-top instruments.

The sensor, depicted in Fig. 1 (a), consists of a cylindrical chamber (designed using Solid Edge by Siemens Systems and fabricated with a MakerBot Replicator 3D printer) devoted to host the 25ml polystyrene vial containing the emulsion between a suitable aqueous reagent (discussed in section 2.2) and the oil sample. The chamber features two diametrically opposed structures hosting a LED, used as light source and a photodiode to detect the light transmitted through the sample. In the case of peroxide value, the LED has a peak emission at 569 nm wavelength (biased with a 30 mA current), while the photodiode is a BPW21R by Vishai (with wavelength peak sensitivity at 565 nm). In the case of total phenol content, instead, the LED has a peak emission at 835nm (biased with a 80 mA current) and the photodiode is a OSD5-5T device by Centronic, with wavelength peak sensitivity between 700 and 900 nm. As discussed in Section3, both the LED peak wavelengths have been chosen by means of preliminary measurements on phenolic and peroxide compounds using a SmartSpec 3000 spectrophotometer.

The experimental set-up is presented in Fig 1 (b). A DC power supply Agilent E3631A is used to provide the LED operating current (I_{LED}) and the power supply for the operational amplifier. The

105 photodiode current (I_{photo}), related to the detected light intensity, is converted into a voltage (V_{out})
106 by a current-to-voltage converter. The voltage V_{out} is acquired by a NI USB-6211 Data Acquisition
107 (DAQ) board by National Instruments and transmitted to a PC for further analysis. All the software
108 for DAQ control, analysis, data presentation and filing has been realized with LabVIEW (National
109 Instruments). Statistical analysis on the experimental data has been carried out with Microsoft
110 EXCEL.

111 *2.3 Chemicals and media*

112 Phenolic reference standards (oleuropein, tyrosol, hydroxytyrosol, *p*-coumaric acid) and peroxide
113 compounds (hydrogen peroxide, H_2O_2, and tert-butyl hydroperoxide, tBuOOH) were purchased
114 from Sigma-Aldrich (St. Louis, MO, USA). The reagent for peroxide value determination was
115 prepared diluting 8 mL of ferrous ion oxidation xylenol orange (FOX) reagent (an aqueous solution
116 of ferrous ammonium sulphate, sorbitol, sulphuric acid and xylenol orange, Sigma-Aldrich)
117 (Cheeseman, 2006) in 7mL of distilled water. The reagent detects the peroxides concentration by
118 oxidation of ferrous ions Fe^{2+} to Fe^{3+} according to the following reaction:

119 $$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO + OH^{-}$$

120 Fe^{3+} ions formed in the reaction are then detected using the dye xylenol orange which binds Fe^{3+}
121 forming a complex that strongly absorbs in the wavelength range 540-580 nm.

122 For the total phenol content, instead, the reagent was prepared mixing: 13 mL of distilled water, 1
123 mL of Folin-Ciocalteu reagent (a mixture of phosphomolybdate acid $H_3PMo_{12}O_{40}$ and
124 phosphotungstate $H_3PW_{12}O_{40}$) and 1 mL of sodium carbonate (Na_2CO_3) 15% (i.e. 15g di sodium
125 carbonate in 100mL of distilled water). As a consequence of the reaction with the phenolic
126 compounds, the acids are reduced to tungsten and molybdenum oxides (W_8O_{23} and Mo_8O_{23})
127 featuring a typical blue colour.

128 In both cases, the reagent was then mixed with 0.5 mL of the oil sample, all stirred for 30 seconds
129 to create the emulsion, then the vial is placed in the sensor for the measure.

All the chemicals used in the experiments are of analytical grade. The olive oil samples used in the experiments were purchased by local markets as well as olive oil mills.

2.4 Reference methods

Olive oil peroxide value has been determined by European standard reference method with starch as indicator and sodium thiosulphate ($Na_2S_2O_3$) as titrant, while total phenol content has been determined according to spectrophotometric method proposed by Singleton and Rossi (Singleton and Rossi, 1965). Phenolic fraction has been extracted using about 4 g of virgin olive oil (VOO) with 5 mL of methanol:water (60:40). The extraction procedure has been repeated two time and the hydroalcoholic fractions have been combined and evaporated by rotavapor up to complete dryness. The concentrated extract has been dissolved in 5 mL of aqueous methanol (50%), and filtered through Minisart RC15 (0.2 μm) regenerated cellulose syringe filters (Sartorius AG, Göttingen, D). Total phenol content has been determined using the Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) and measuring at 750 nm with a Shimadzu spectrophotometer UV-VIS 1204 (Kyoto, Japan). The results have been expressed as mg of gallic acid per kg of VOO (gallic acid calibration curve R^2= 0.993).

3. Results and discussion

The reagents response was initially characterized with peroxide and phenolic compounds using a SmartSpec 3000 spectrophotometer. Then a set of 25 olive oil samples have been analyzed with the technique and the bench-top set-up described in Section 2.1. Finally an electronic board has been designed and fabricated to avoid the use of all bench-top instrumentation, thus demonstrating the feasibility of a simple and economical instrument for easy, fast and in-situ analysis of olive oil.

3.1. Spectral characterization of reagents response

At first, the reagents were inoculated with known concentrations of peroxide and phenolic compounds and the absorption spectra acquired with a SmartSpec 3000 spectrophotometer.

In the case of phenols, four different compounds were tested: three of these are typically present at high concentration (oleuropein, hydroxytyrosol and tyrosol) while the forth one (*p*-coumaric acid) is present in small concentration. Fig. 2 (a) shows the absorption spectra in the case of different concentrations of oleuropein in the 400 – 800 nm wavelength range. As can be seen the different concentrations of phenolic compound can be discriminated for wavelength > 500 nm with the sensitivity increasing with the wavelength. In Fig. 2 (b) the absorbance measured at the wavelength of 800 nm is plotted vs. the phenol content for all the tested compounds. Except for tyrosol for which a higher sensitivity for concentrations below than 15 ppm is registred, in all cases a linear relation between absorbance and concentration is found. The linear regression lines of the four cases are calculated to estimate the compound sensitivity (Abs800/ppm) to the reagent with the following results: 0.0199 (oleuropein), 0.1159 (hydroxytyrosol), 0.0248 (*p*-coumaric acid), while in the case of tyrosol the sensitivity is 0.0755 for concentrations lower than 15 ppm and 0.019843 for concentrations higher than 15 ppm. As depicted in Fig 2 (b), the intensity of blue color obtained by reduction of tungsten and molybdenum oxides is proportional to the concentration of each phenol but shows a different response respect to the phenol structure which reacted. In fact Folin-Ciocalteu detects the total reducing capacity that is different for different compounds (Apak et al., 2007). As described in several paper, during the storage of VOO the trend of phenolic fraction, measured by Folin-Ciocalteu assay showed a fluctuation due to the oxidation and lysis of phenolic compounds disperse in the matrix. This result confirms the different response factor that the reaction mixture presents towards individual phenols (Boselli et al., 2009; Fiori et al., 2014).

In the case of peroxides, two different compounds were tested: hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (tBuOOH). The absorbance spectra in the wavelength range 400 - 800 nm are shown in Fig. 3 (a) for different tBuOOH concentrations. As expected, the reaction of Fe^{2+} ions with peroxide compounds results in the formation of Fe^{3+} ions that bind to xylenol orange producing an absorbance peak between 560 and 580 nm. The measured absorbance at 580 nm is plotted vs. compound concentration for both H_2O_2 and tBuOOH in Fig. 3 (b). From the measured

181 data, the absorbance can be empirically modelled as a linear function of the square root of the
182 compound concentration. The results indicate that H_2O_2 ($y = 0.297\sqrt{x} + 0.436$) is characterized by
183 higher sensitivity than tBuOOH ($y = 0.115\sqrt{x} + 0.265$). In both cases a determination coefficient R^2
184 higher than 0.96 is achieved. The different response of FOX reagent to H_2O_2 and tBuOOH can be
185 due to the ability of sorbitol to scavenge hydroxyl radicals to yield peroxyl radicals which would
186 propagate Fe^{2+} oxidation (Jiang et al., 1990).

187 *3.2. Experimental results*

188 A set of 25 olive oil samples have been tested using the technique and the experimental set-up
189 described in Section 2. The voltage V_{out} is acquired at time intervals of 5 seconds for a total of 1200
190 seconds for both tests (total phenol content and peroxide value).

191 In the case of total phenol content, the logarithm of V_{out} has been found to be linearly related with
192 the phenols concentration. The coefficient of determination R^2 has been calculated after different
193 time intervals and plotted as function of time in Fig. 4 (a): the correlation between $Log_{10}(V_{out}/V_M)$
194 (where V_M is the measured voltage in the absence of oil sample, i.e. due to the reagent only) and the
195 total phenol content increases with time, reaching a plateu after 600 seconds. Fig. 4 (b) shows the
196 measured values of $Log_{10}(V_{out}/V_M)$ after 600 seconds plotted vs total phenol content as determined
197 using the reference method. The linear regression line equation is $Y = 0.0343 - 2.389 \cdot 10^{-3} \cdot X$ and the
198 coefficient of determination 0.895. The accuracy is slightly higher to what reported by Cerretani
199 (Cerretani et al., 2010) when a R^2 of 0.87 was obtained by FTIR attenuated total reflectance
200 spectroscopy using a wavelength range 3610 – 816 cm^{-1} and a PLS chemometric analysis. The use
201 of an array of voltammetric sensors (Rodriguez-Mendez et al., 2008) reported an higher accuracy
202 ($R^2 = 0.986$), but the samples tested were only 6 olive oils and all above the 400 ppm; instead, in the
203 present work, 25 samples were analysed and the level of detection achieved was of 100 ppm.

204 In the case of peroxide value, a linear relation between the measured values of V_{out}/V_M and the
205 peroxide value has been found. As shown in Fig. 4 (c), the coefficient R^2 increases with time
206 reaching a plateau after about 600 seconds . Fig. 4 (d) shows the measured values of V_{out}/V_M plotted

vs. the peroxide value as determined by the reference method. The linear regression line equation is $Y = 0.6368 - 13.8 \cdot 10^{-3} \cdot X$ and the coefficient of determination 0.883. The dispersion between measured data and the reference technique can be mainly associated with the property of the FOX reagent that is able to determine all kinds of conjugated dienes including those without a peroxide group, and not able to react with peroxides included in molecules without conjugated double bonds. Furthermore the accuracy of the quantification can be also diminished by the presence of several other compounds present in olive oil, such as carotenoids and chlorophyll, absorbing at 500-600 nm, and chain-breaking antioxidant able to reduce the color yield (Bou et al 2008). The obtained accuracy is however higher than that reported in (Armenta et al., 2007) where a coefficient of determination R^2 of 0.6558 resulted by NIR spectroscopy combined with PLS chemometric analysis.

In both cases there is a linear relation between the parameter measured with the experimental set-up ($Log_{10}(V_{out}/V_M)$ for total phenol content and V_{out}/V_M for peroxide value) and the oil quality parameter measured with the reference methods. Thus, using the equation of the linear regression line, the oil quality parameters can be estimated.

3.3. Implementation as a portable embedded system

To demonstrate that the technique proposed in this work can lead to a portable embedded system suitable for in-situ measurements, all the operations performed by the bench-top instrumentation and DAQ PC board have been implemented inside an electronic board designed “ad hoc”, based on the µcontroller Dspic33ep512mu810, whose schematic is presented in Fig. 5 (a).

The LED is supplied with a square wave current (frequency 1 kHz) to allow removing the contribution of the environment radiation. In particular, a square wave voltage ranging from 0 to 830 mV is generated by the µcontroller and fed to the noninverting input of an operational amplifier. In turn, this latter drives a BJT transistor providing the LED with the supply current I_{LED} (selectable with the value of the resistance R_{LED}). The radiation transmitted through the sample is received by the photodiode that generates the current I_{photo} proportional to the incident radiation. An

I/V converter generates a voltage V_A proportional to I_{photo}. A couple of programmable switches allows to select two different feedback resistors (R_{F1} or R_{F2}). The voltage V_A is then fed to a AC coupling stage that generates a square wave voltage (V_B) with mean value 2.5V. Such a voltage is then fed to a synchronous rectifier (built with an AD8271 difference amplifier and an ADG733 triple single pole double throw SPDT switch) that provides a DC voltage (V_C) equal to the high level of V_B. Then, a 18 bit ADC driver ADA4941 generates a differential voltage V_{OUT} (proportional to V_C) that is fed to a 12 bit ADC (AD9220AR) providing the 12 bit digital codification of V_{OUT} (D_{OUT}) to be processed by the μcontroller. The measured data are sent to a portable PC (via USB interface) to display the results and file the data. The system has been built by using low-cost electronics and its total cost has been estimated in about 300 $. All the software has been developed using LabVIEW. Fig. 5 (b) and (c) present pictures of the sensor, while Fig. 5 (d) is a photograph of the electronic board.

A significant subset of the olive oil samples have been tested using both the bench-top instrument set-up described in Section 2.1 and the embedded system presented above to compare the results.

Fig. 6 (a) shows a plot of $Log_{10}(D_{OUT}/4096)$ vs. time (acquisitions every 5 seconds) for 3 samples featuring different values of total phenol content (the division by 4096 represents a normalization necessary for comparison with V_{out}/V_M of sub-Section 3.1). As can be seen, higher total phenol content results in lower values of measured D_{OUT}. Measurements on a set of 10 olive oil samples result in a linear correlation between $Log_{10}(D_{OUT}/4096)$ and total phenol content ($Y = -4.978 \cdot 10^{-4} \cdot X - 4.7 \cdot 10^{-3}$).

In Fig. 6 (b) $D_{OUT}/4096$ is plotted vs. time for 3 samples featuring different values of peroxide value and, as can be seen, more oxidized samples exhibit lower values of D_{OUT}. Measurements on a set of 7 olive oil samples result in a linear correlation between $D_{OUT}/4096$ and the peroxide value ($Y = 8.532 \cdot 10^{-3} \cdot X + 0.893$).

The linear regression equations have been used to compare the estimated values of both peroxide value and total phenol content with those obtained with the reference methods and the calculated

259 determination coefficient R^2 differs by less than 1.6% between the two measuring systems. In
260 particular, in the case of peroxide value, R^2 = 0.901 and 0.887 for the bench-top set-up and the
261 embedded system, respectively; while with total phenol content R^2 = 0.932 and 0.922 for the bench-
262 top set-up and the embedded system, respectively. The agreement between the two measuring
263 systems is very good.
264
265 **4. Conclusions**
266 A novel technique to measure peroxide value and total phenol content in olive oil has been
267 presented that is based on optical density measurements of a suitable reagent inoculated with the
268 olive oil of interest. The technique, suitable to be realized in the form of a low-cost, embedded
269 electronic system, has been tested using an experimental set-up built with bench-top
270 instrumentation and the results show that it can estimate with good accuracy the peroxide value (R^2
271 = 0.883) and the total phenol content (R^2 = 0.895) of olive oil in less than 10 minutes.
272 The proposed technique has been implemented in an electronic board to realize a portable
273 embedded system; the results obtained with such an instrument have been compared with those
274 coming from the bench-top set-up and an excellent agreement has been found.
275 The portable embedded system could be applied for fast and *in-situ* olive oil quality control and to
276 assess the shelf-life without the need of trained personnel. Furthermore, it could be useful for a first
277 evaluation of the total phenol content of virgin olive oils in case of interest of a productor to report
278 on the lable the oilive oil claim, according with the EU Regulation 432/2012, stating that “Olive oil
279 polyphenols contribute to the protection of blood lipids from oxidative stress”, but admitted only if
280 at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) are
281 contained in 20 g of olive oil.
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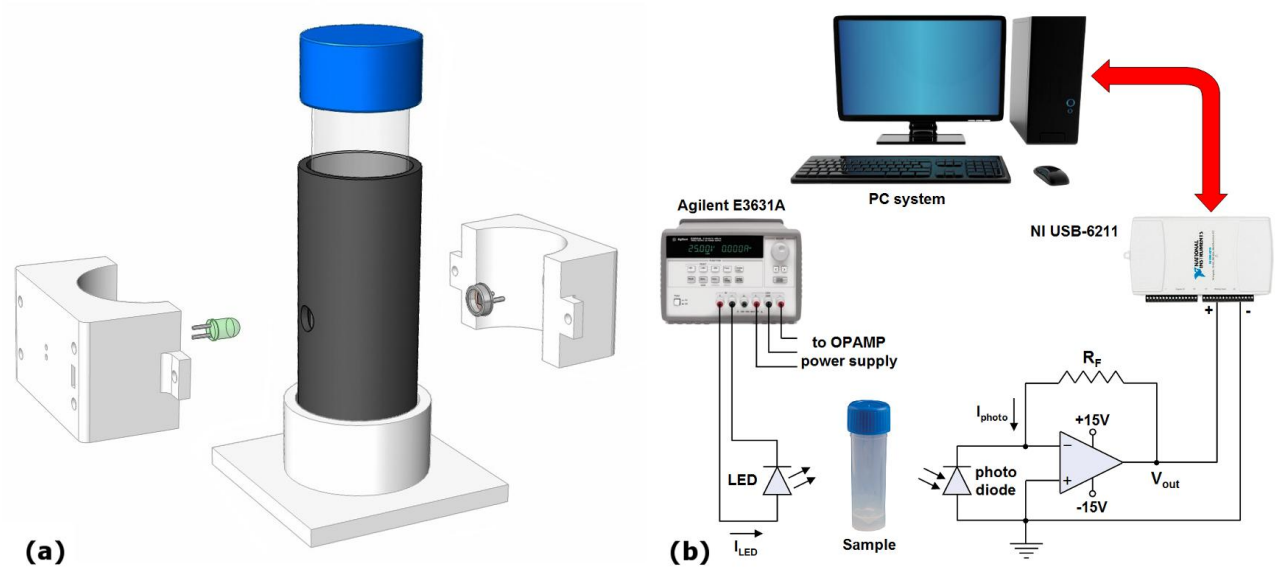

Fig. 1. The sensor used for the experiments of the present work (a) and the experimental set-up built with bench-top instrumentation used for the measurements (b).

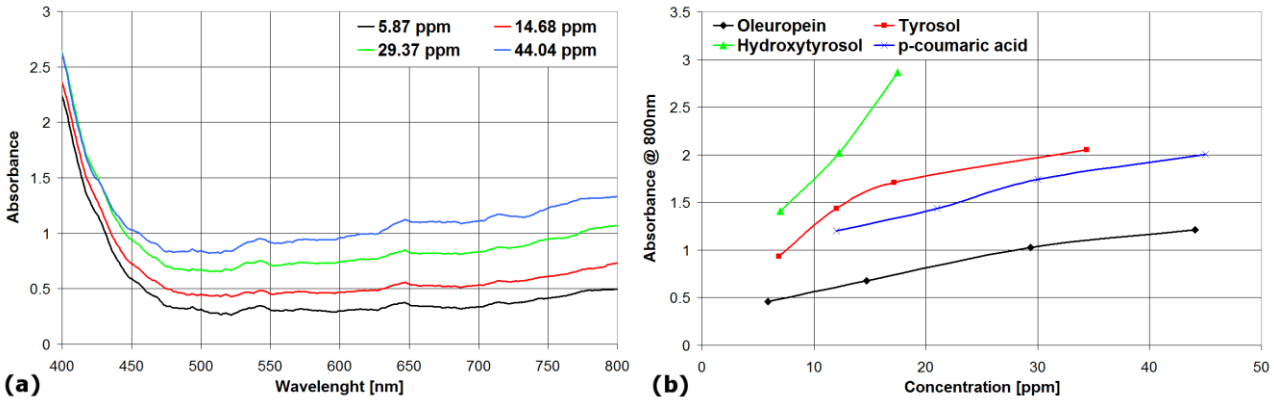

Fig. 2. Measured absorbance vs. wavelength in the range 400 – 800 nm for different concentrations of oleuropein (a) and measured absorbance at 800 nm plotted vs. phenol content for different phenolic compounds (b).

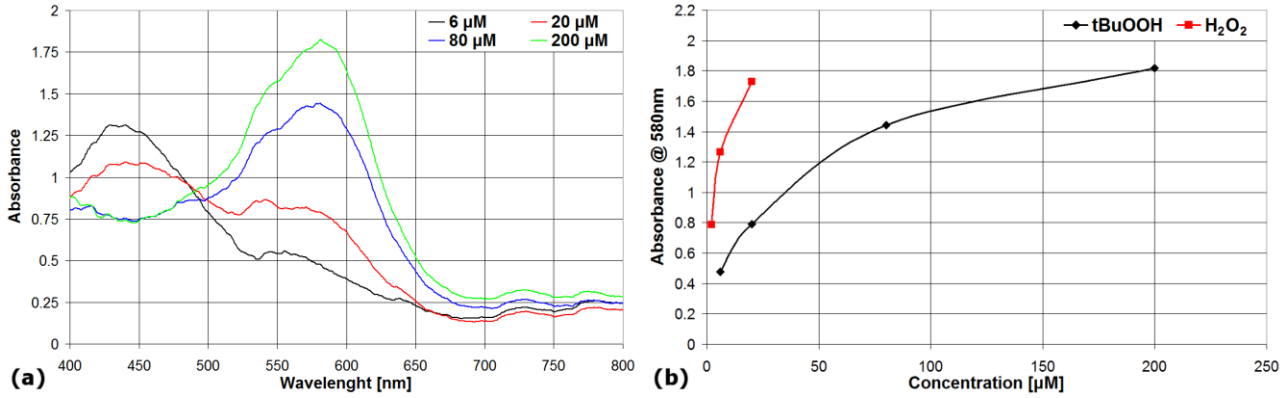

Fig. 3. Absorbance plotted vs. wavelength in the range 400 – 800 nm for different concentrations of tert-butyl hydroperoxide (a) and measured absorbance at 580 nm plotted vs. concentration for tert-butyl hydroperoxide as well as vs. hydrogen peroxide (b).

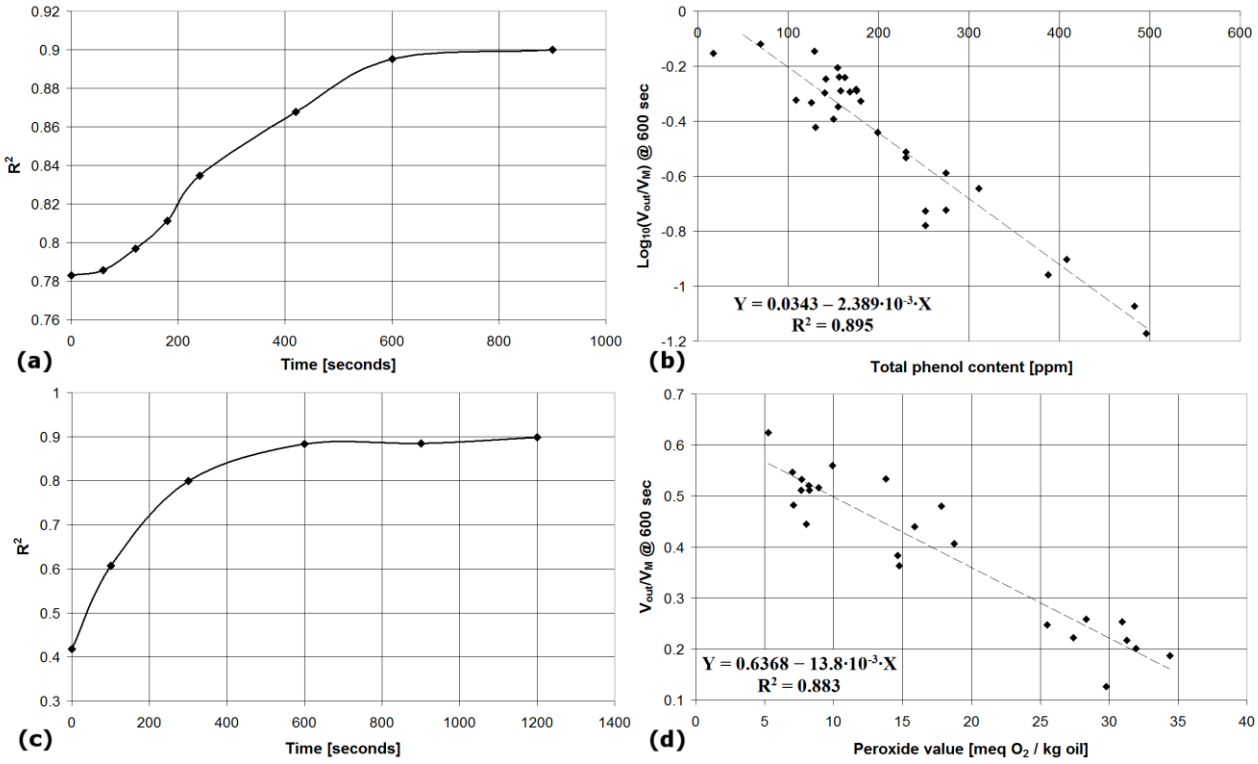

Fig. 4. Coefficient of determination R^2 plotted vs. time for the total phenol content (a) and peroxide value (c). Scatter plot and linear regression line calculated for 600 seconds for total phenol content (b) and peroxide value (d).

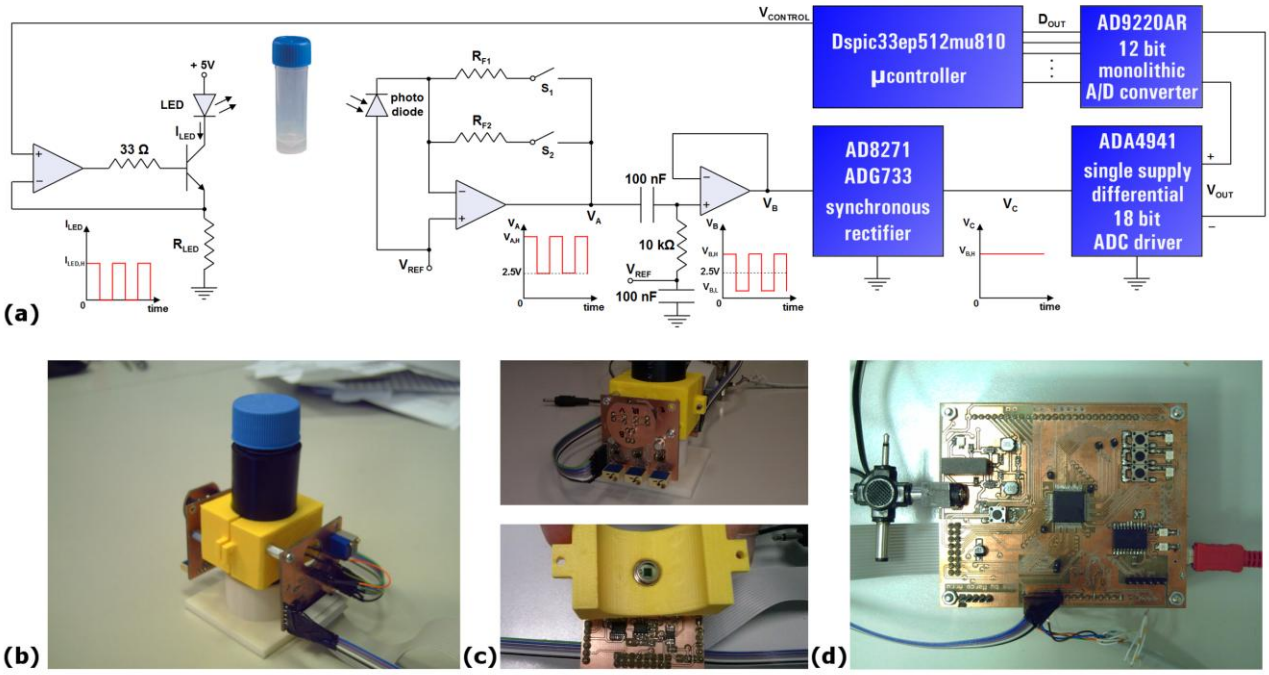

Fig. 5. Schematic of the electronic board of the embedded system realized in the present work (a); pictures of the sensor (b) and (c); photograph of the electronic board (d).

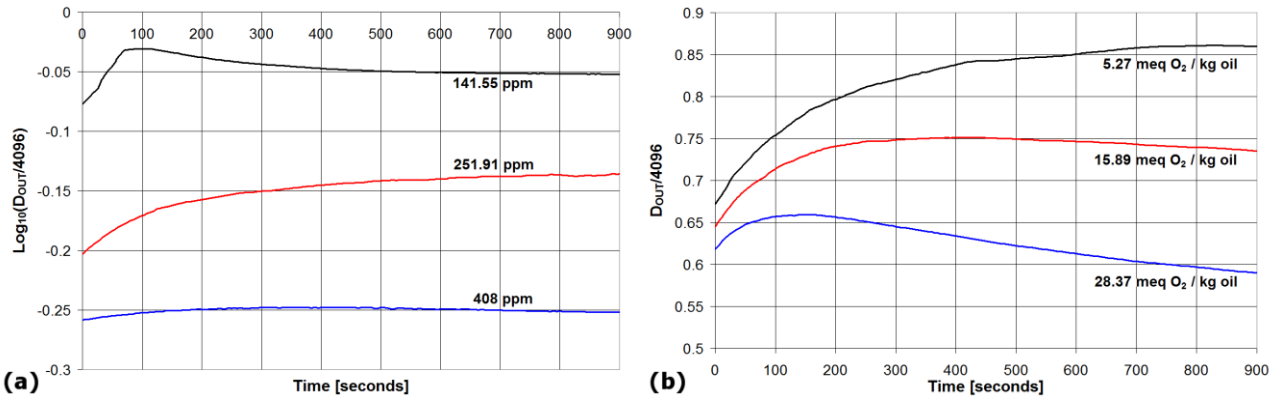

Fig. 6. Data measured with the designed electronic board for different oil samples vs. time in the case of total phenol content (a) and peroxide value (b).