**A SIMPLE ONE-POT DETERMINATION OF BOTH TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF HONEY BY POLYMER CHEMOSENSORS**

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**ABSTRACT**

We have developed a new method for the rapid (2h) and inexpensive (materials cost<0.02 €/sample) “2-in-1” determination of the total phenolic content (**TPC**) and the antioxidant activity (**AOX**) in honey samples. The method is based on hydrophilic colorimetric films with diazonium groups, which react with phenols rendering highly colored azo groups. The ***TPC*** of the sample is correlated to its trolox equivalent antioxidant capacity (***TEAC***). The intensity of the color allows us to determine both ***TPC*** and ***TEAC*** of the sample by the analysis of a picture taken with a smartphone that is analysed by the use of the color-definition-parameters (RGB). The controlled light conditions and the systematic use of the same camera avoid the periodical calibration of the system improving the efficiency of the method. Thus, it is a simple method carried out by non-specialized personnel and it involves much lower money and time investment compared to traditional methods.

**KEYWORDS**: Honey; sensor; total phenolic content; antioxidant activity; TEAC; polymer; polyphenols

1. **Introduction**

Honey is a product made by bees from flower nectar or honeydew mixed with bees’ secretions after maturing in honeycombs (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). It is used as a natural sweetener. Honey contains more than 181 compounds, being the monosaccharides fructose and glucose the most abundant ones. The proportion of each sugar is different, depending on the honey type (Ball, 2007). Honey has also low quantities of other compounds, such as proteins, enzymes, vitamins, amino acids, minerals, trace elements, aromatic substances, and polyphenols (Bogdanov, 2016). Currently, honey polyphenols are of growing interest because they help authenticate honey, and notoriously contribute to antioxidant capacity, antimicrobial activity (Alvarez-Suarez et al., 2010; Baltrušayt, Venskmonis, & Čeksteryte, 2007; Estevinho, Pereira, Moreira, Dias, & Pereira, 2008; Rao, Krishnan, Salleh, & Gan, 2016), as well as other potentially beneficial effects.

In 2017, the world production of honey was around 1,800,000 t, being more than 700,000 t intended for international trade with an estimated export value of 2,364 million US$ (FAOSTAT, 2014). Spain ranked in the 4th worldwide position, leading the EU honey production with 29,393.2 t (Ministerio de Agricultura Pesca y Alimentación, 2019). All these data show the commercial significance of honey, so that researching on new rapid and low cost methods for its authentication and quality control is of utmost importance. 

Apart from the compulsory parameters of legal regulations (Thrasyvoulou et al., 2018), nowadays, most laboratories that analyze honeys determine both total phenolic content (**TPC**) and antioxidant activity (**AOX**), because the results of these parameters can contribute to improve honey commercialization. The most common method to analyze **TPC** is the spectroscopic assay using the Folin-Ciocalteu reagent (Singleton & Rossi, 1965), that is still time consuming despite having been successively modified and improved. As for the measurement of **AOX**, one of the most employed procedures is the spectrophotometric method known as trolox equivalent antioxidant capacity (***TEAC***), using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (***ABTS***) as the radical source (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999), that is also time-consuming, because the ***ABTS*** radical formation takes 16 hours. Other methods as GS/MS or HPLC with different detectors require a large expenditure of money and time, as well as specialized personnel (Quintero-Lira, Ángeles Santos, Aguirre-Álvarez, Reyes-Munguía, Almaraz-Buendía, & Campos-Montiel, 2017). For all this, a quick, easy, and simple method for the double detection of **TPC** and **AOX** of honey would be a breakthrough in this field.

Chemical sensors (or chemosensors) are rapid detection methods, based on the interaction of a receptor molecule with target species, which generates a quantifiable change in a macroscopic property of the material. In the case of a change of color (chromogenic sensor), the response can be visually for a semi-quantitative detection but can be also easily registered and analyzed with a smartphone and a PC respectively, by using the digital color definition parameters (RGB) (Vallejos, Muñoz, Ibeas, Serna, García, & García, 2013). The use of chemosensors for honey control is increasingly being used, mainly concerning honey sugars (Isa et al., 2017; Revenga-Parra et al., 2020), and antibiotics’ residues detection (Bougrini et al., 2016; Cervera-Chiner et al., 2020).

The purpose of this work is to develop sensory colorimetric films for the rapid and low cost dual determination of the **TPC** and the **AOX** in honey samples, so that the analysis can be carried out by non-specialized personnel, quickly, and without using dangerous reagents, organic solvents, or personal protective equipment (PPE).

1. **Materials and Methods**
   1. *Samples*

This study was carried out with eight representative honeys harvested in 2019 in Castilla-León, a Spanish area located in the northern Iberian Plateau that held the highest number (3,827) of apicultural undertakings in Spain in 2018, representing 16% of the total apicultural undertakings of this country (Ministerio de Agricultura, Pesca y Alimentación, 2019). Honeys’ botanical origins were determined by both melissopalinology (Louveaux, Maurizio, & Vorwohl, 1978; Terradillos, Muniategui, Sancho, Huidobro, & Simal-Lozano, 1994; Von Der Ohe, Persano Oddo, Piana, Morlot, & Martin, 2004), and sensory analyses (Marcazzan, Mucignat-Caretta, Marina Marchese, & Piana, 2018; Persano Oddo & Piro, 2004; Piana, Persano Oddo, Bentabol, Bruneau, Bogdanov, & Guyot Declerck, 2004), there being 1 ling heather (*Calluna vulgaris* (L.) Hull) honey (sample 1), 2 honeydew honeys (samples 2 and 3) and 5 multifloral honeys (samples 4-8). The sediment of the samples showed that the most important secondary pollen types were Leguminosae type *Trifolium* spp., Leguminosae type *Genista* spp., Rosaceae type *Rubus* spp. and Compositae type *Helianthus annuus* L. Additional information about honey samples in **ESI S1.**

*2.2. Materials*

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: sodium hydroxide (VWR, 99 %), 1-vinyl-2-pyrrolidone (**VP**) (Aldrich, ≥ 98%), methylmethacrylate (**MMA**) (Aldrich, 99%), 4-aminostyrene (**SNH2**) (Aldrich, 97%), sodium nitrite (VWR, 99.5%), m-cresol (AlfaAesar, > 99 %), Folin-Ciocalteu 2 N reagent solution (Aldrich, 99%), hydrochloric acid (Aldrich, 37 %), gallic acid (**GA)** (Aldrich, 97.5-102.5 %), sodium carbonate (Aldrich, 99.5 %), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (***ABTS***) (Aldrich, ≥ 98 %, HPLC), potassium persulfate (Aldrich, ≥ 99%), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) (Aldrich, 97%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallized twice from methanol.

For the ***Folin-Ciocalteu*** and ***TEAC*** methods, honeys were treated according to the following procedure: 5 g honey was dissolved in 10 mL of distilled water. Then, the solution was transferred to a 50 mL flask, completed with distilled water, and filtered through a Whatman nº1 paper. The resulting solution was labeled as **HOx**. For the method of sensory colorimetric films, 5 g honey was dissolved in 45 ml NaOH aqueous solution (0.1M). The mixture was shaken and homogenized for 10 minutes and then, it was filtered through a Whatman nº1 paper. The resulting solution was labeled as **B-HOx**. More information about honey samples in **ESI S1.**

*2.3. Preparation of the sensory polymeric films*

The starting film was prepared by bulk radical polymerization of three commercial monomers: **VP**, **MMA**, and **SNH2** in a molar feed ratio of 49.5/49.5/1 (**VP**/**MMA**/**SNH2**) using 1% mol of AIBN as radical thermal initiator. The polymerization was carried out at 60 ºC, overnight, in a mold comprised between two silanized glasses (100 µm thick), in an oxygen-free atmosphere. The film was removed from the mold and 8 mm diameter discs were cut with a punch and dipped into an acid solution of NaNO2 (10 mL of water, 1 mL of HCl 37 %, and 40 mg of NaNO2) at RT for 90 min.. Stable sensory films with pendant benzenediazonium salt motifs were easily prepared by this way.(Bustamante et al., 2019)

*2.4. Instrumentation and methods*

UV/Vis spectra were recorded using a Hitachi U-3900 UV/Vis spectrophotometer. The RGB analysis was carried out by taking a picture of the polymeric discs, with a Samsung Galaxy S8 smartphone and a retro-illumination homemade lightbox previously developed,(Vallejos, Reglero, García, & García, 2017, stereolithography archive available as supporting data) essential to always reproduce the same light and distance conditions (camera specifications: 1/3.6″ 8Mp sensor-1.22µm pixel size, f/1.7-aperture lens, 25mm-equivalent focal length, autofocus). The sample tubes used for the preparation of the different honey solutions (**HOx**) shaking, were carried out using a vortex shaker (VWR).

The reference method for the determination of **TPC** of honey was the so-called ***Folin-Ciocalteu*** assay, and it was performed according to the methodology described by Sancho et al. (2016), based on Singleton & Rossi (1965) procedure*.* To carry it out, 0.5 mL of **HOx** was mixed with 2.5 mL of ***Folin-Ciocalteu*** 0.2 N reagent solution. The resulting mixture was homogenized using a tubes agitator (Vortex shaker, VWR) and then, the samples were left at room temperature for 5 minutes and 2 mL of sodium carbonate (75 g/L) was added and the mixture was homogenized again. Finally, the tubes were incubated for two hours in darkness at room temperature and the absorbance of samples was recorded at 760 nm using a Hitachi U-3900 UV/Vis spectrophotometer. More information about this method in **ESI S3.**

The chosen reference method for determining the antioxidant activity was the ***TEAC*** method (Re et al., 1999), modified for our purpose (Sancho et al., 2016), based on the inhibition of ***ABTS*** by honey components. To carry out this assay, 10 µl of **HOx** was mixed with 1490 µl of ***ABTS*** radical (***ABTS*** radical is generated by mixingan aqueous solution of ***ABTS*** (7 mM), and K2S2O8 (2.45 mM) aqueous solution. The resulting solution was stirred in darkness for 16-18 h at room temperature. Secondly, 1490 µl of ***ABTS*** reagent was mixed with 10 µl of standard trolox aqueous solutions ranging from 0 to 3 mM, and the absorbance at 734 nm was recorded after 6 minutes (A6) by triplicate. The inhibition percentage of the sample was obtained by the following equation (**100\*(Ab-A6)/Ab**), where **Ab** is the absorbance (734nm) of the sample used as blank and **A6** the absorbance of the mixture of **ABTS** and **Trolox** sample after 6 minutes mixed). The resulting mixture was homogenized and then, the samples were incubated for 6 minutes in darkness at room temperature and the absorbance of samples was recorded at 734 nm using a Hitachi U-3900 UV/Vis spectrophotometer. More information about this method in **ESI S4.**

For the analysis with the sensory colorimetric films, 8 mm diameter discs were directly dipped for 2 hours in 10 ml of **B-HOx** at room temperature, without a further experimental procedure. The discs were removed from the solution and washed 3 times with NaOH 0.1M for 15 minutes for finally taking the photographs in triplicate. RGB parameters of the pictures were analyzed using GIMP 2 free image software, and it was found that the B (blue) parameter is the only significant variable, the only one that brings relevant information. In the same way, a titration of the sensory colorimetric films with gallic acid (**GA**) was carried out, by dipping 8 mm diameter discs in basic aqueous solutions (NaOH 0.1M) of **GA** ranging from 50 to 260 mg/L.

1. **Results and discussion**

The sensory polymers that the study is about have been already tested and characterized as sensors for different kinds of phenols, as phenols used as fungicides and pesticides (Bustamante et al., 2019), or polyphenols in wines (Vallejos, Moreno, Ibeas, Muñoz, García, & García, 2019). In this case, we have oriented the developed materials for a real need, improving the existing reference methods for the determination of **TPC** and **AOX** in terms of time, money, and simplicity. For that, honeys were firstly analyzed with the reference methods, and secondly with the proposed one.

*3.1. Titration of sensory colorimetric films with* ***GA*** *using the sensory colorimetric films*

**GA** is the phenol used as a standard in ***Folin-Ciocalteu*** reference method. In our previous works (Bustamante et al., 2019), this sensor has been tested with various phenols, but not with **GA**, so we found mandatory to confirm the sensory behavior of the sensory colorimetric films with this phenol. However, this is only a verification test, which does not take in account the matrix effect of honey.

The titration with **GA** was performed by dipping 8 mm sensory discs in aqueous basic solutions (NaOH, 0.1 M) of **GA** in concentrations ranging from 50 to 260 mg/L (**Figure 1**) following the procedure and washing process described in the experimental part. The color development of the sensory films from grey to brow-yellowish could be visually broadly correlated with the **GA** concentration. The titration curve was built correlating the blue (B) parameters of the digital images of the films with the concentration of **GA**, as depicted in **Figure 1** (RGB parameters of the digital photographs are shown in **ESI, Section S2**). This Figure shows a linear (R2 = 0.994) correlation pointing out to the sensory films as tools to detect and measure the concentration of **GA** in water environments. Therefore, and based on this result, the next step was to measure the **TPC** in honey samples with the sensory polymeric films comparing, at the same time, the results obtained with ***Folin-Ciocalteu*** method.

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| **Figure 1.** *Top:* Pictures were taken to the 8 mm diameter sensory films after dipping for 2h in NaOH 0.1M solutions of **GA** in different concentrations; from 50 to 260 mg/L. *Graph:* Graphical representation of the blue parameter (B) of the RGB parameters *vs* the concentration of **GA**. The fitted linear equation is showed within the graph. |

*3.2. Determination of the total phenolic content (****TPC****) of honeys by* ***Folin-Ciocalteu*** *method*

This spectrophotometric method measures the absorption of a blue complex formed through a redox reaction between a sample´s phenols and the ***Folin-Ciocalteu*** reagent, as compared with a **GA** standard (Gülçin, Şat, Beydemir, Elmastaş, & Küfrevioǧlu, 2004). The **TPC** of honeys is expressed as mg of **GA** per 100 grams of honey. **Table 1** shows the results for the measured honey samples, and the corresponding titration curve with **GA** can be found in **ESI S3.**

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| **Table 1.** Total phenolic content of the measured honeys by the ***Folin-Ciocalteu*** method. The table shows the average of the 3 measurements of each honey sample. | |
| **Total phenolic content**  **(mg GA / 100 g honey)** | |
| Honey-1 | 106.71±4.87 |
| Honey-2 | 37.44±3.25 |
| Honey-3 | 120.90±5.18 |
| Honey-4 | 46.85±3.47 |
| Honey-5 | 30.16±3.09 |
| Honey-6 | 53.91±3.63 |
| Honey-7 | 93.68±4.56 |
| Honey-8 | 35.72±3.21 |

*3.3. Determination of antioxidant activity by* ***TEAC*** *assay*

The method follows the suppression of the characteristic long-wave absorption band of the ***ABTS*** radical in presence of hydrogen-donating antioxidants. This spectrophotometric assay measures the relative ability of antioxidants to scavenge the ***ABTS*** radical generated in aqueous phase, as compared with a trolox (water-soluble vitamin E analog) standard (Dykes, Rooney, Waniska, & Rooney, 2005), thus, the antioxidant activity is usually expressed as mol of trolox per 100 grams honey. **Table 2** shows the results for the measured honeys (fresh weight), and the corresponding titration curve with trolox can be found in **ESI S4.**

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| **Table 2.** Antioxidant activity of the measured honey by the ***TEAC*** method. The table shows the average of 3 measurements of each honey, and the obtained antioxidant activity expressed as mol of trolox / 100 g honey. | |
| **Antioxidant activity**  **(µmol trolox / 100 g honey)** | |
| **Honey-1** | 729.33±116.02 |
| **Honey-2** | 459.53±104.81 |
| **Honey-3** | 727.39±115.94 |
| **Honey-4** | 485.84±105.90 |
| **Honey-5** | 395.83±102.16 |
| **Honey-6** | 470.66±105.27 |
| **Honey-7** | 615.07±111.27 |
| **Honey-8** | 447.48±104.30 |

*3.4. Correlation study between* ***Folin-Ciocalteu*** *and* ***TEAC*** *methods with the sensory colorimetric films.*

The proposed method of the sensory colorimetric films is based on the color change produced by the formed highly colored azo groups between a sample´s phenols and the diazonium salt motifs of the discs. The measured experimental variable is the blue parameter of the RGB parameters (see **ESI S5**), and is represented *vs* the obtained data from ***Folin-Ciocalteu*** and ***TEAC*** methods. The correlation between methods is clearly observed in **Figure 2**, and the initial proposal to determine both the **TPC** and the **AOX** with a single analysis is confirmed, just by dipping the sensory colorimetric films for 2 hours in **B-HO(1-8)** solutions at room temperature. This new method saves time, reagents, and money in comparison, not only with ***Folin-Ciocalteu*** or ***TEAC*** methods but also with others which present the same drawbacks. **Table 3** shows a brief comparison of all these methods with our proposed one, in terms of required equipment, response time, visual response (Y/N), and low-cost nature (Y/N).

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| **a)** |
| **b)** |
| **c**) |
| **Figure 2.** **a)** Sensory colorimetric films after dipping in 10 ml of **B-HO(1-8)** solutions for 2 hours at room temperature; **b)** Correlation between total phenolic content obtained by ***Folin-Ciocalteu*** method and B parameter of the sensory colorimetric films (B parameter); **c)** Correlation between the antioxidant activity obtained by ***TEAC*** method and B parameter of the sensory colorimetric films. |

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| **Table 3.** Comparison between conventional methods and sensory colorimetric film for the determination of the total phenolic content (**TPC**)and antioxidant activity. | | | | | | |
| **Methodology** | **Equipment** | **Measured property** | **Response time** | **Visual response** | **Low-Cost** | **References** |
| **TPC** | Spectrophotometer.  Vortex shaker. | Polyphenol  content | ≈3.5 h | No | No | (Alvarez-Suarez et al., 2010) |
| ***TEAC* method** | Spectrophotometer,  Vortex shaker. | Antioxidant activity | ≈3 h | No | No | (Alvarez-Suarez et al., 2010; Moniruzzaman, Khalil, Sulaiman, & Gan, 2012) |
| **High-performance liquid chromatography (HPLC)** | HPLC | Polyphenol  content | ≈5 h | No | No | (Pyrzynska & Biesaga, 2009) |
| **Gas chromatography (GC)** | Gas chromatograph | Polyphenol  content | ≈12 h | No | No | (Alvarez-Suarez et al., 2010; Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006) |
| **Capilar electrophoresis (CE)** | Power source, capillaries, detector. | Polyphenol  content | ≈4 h | No | No | (Andrade, Ferreres, Gil, & Tomás-Barberán, 1997; Gómez-Caravaca et al., 2006) |
| **Ultra performance liquid chromatography (UPLC)** | Chromatographic  system | Polyphenol  content | ≈5 h. | No | No | (Alvarez-Suarez et al., 2010; Muñoz Jáuregui, Ortíz Ureta, Blanco Blasco, Castañeda Castañeda, Alvarado Yarasca, & Ruiz Quiroz, 2014; Trautvetter, Koelling-speer, Speer, Trautvetter, Koelling-speer, & Speer, 2009) |
| **FRAP assay** | Spectrophotometer | Antioxidant activity | ≈1.5 h | No | No | (Aljadi & Kamaruddin, 2004; Alvarez-Suarez et al., 2010; Lachman, Orsák, Hejtmánková, & Kovářová, 2010; Moniruzzaman et al., 2012; Muñoz Jáuregui et al., 2014) |
| **DPPH assay** | Spectrophotometer,  Vortex shaker. | Antioxidant activity | ≈1.5 h | No | No | (Akbulut, Özcan, & Çoklar, 2009; Moniruzzaman et al., 2012; Muñoz Jáuregui et al., 2014; Trautvetter et al., 2009) |
| **2-desoxi-D-ribose assay** | Spectrophotometer,  Water bath | Antioxidant activity | ≈3.5 h | No | No | *(Repositorio Institucional Universidad Distrital - RIUD: Estudio Cromatográfico por HPLC-UV, Cuantificación de Fenoles, Flavonoides y Evaluación de la Capacidad Antioxidante en Miel de Abejas*>, n.d.; Trautvetter et al., 2009) |
| **Superoxide anion assay** | Spectrophotometer,  Vortex shaker. | Antioxidant activity | ≈30 min | No | No | (Lachman et al., 2010; Trautvetter et al., 2009) |
| **Polymeric sensor** | Smartphone, PC, Vortex shaker. | Polyphenol  content | 2 h | Yes | Yes | (Vallejos et al., 2019) |
| **Sensory colorimetric films** | Smartphone, PC, Vortex shaker. | Antioxidant activity & Polyphenol  content | 2 h | Yes | Yes | This Work |

*3.5. Proof of concept. Determination of* ***TPC*** *and* ***AOX*** *with sensory colorimetric films.*

Once demonstrated the correlation between the reference methods and the proposed method, is possible to calculate the **TPC** and the **AOX** of all honeys only by substituting B parameter in the fitted equations showed in **Figure 2**. As summary, **Table 4** shows the obtained results of **TPC** and **AOX**, both by reference methods and the proposed one.

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| **Table 4**. Total phenolic content and antioxidant activity of honeys measured both the reference methods (***Folin-Ciocalteu*** and ***TEAC***) and the proposed one (sensory colorimetric films). | | | | |
|  | **Total phenolic content** | | **Antioxidant activity** | |
|  | ***Folin-Ciocalteu***method  (mg GA / 100 g honey) | *Sensory colorimetric films*  (mg GA / 100 g honey) | ***TEAC*** method  (mol trolox / 100 g honey) | *Sensory colorimetric films*  (mol trolox / 100 g honey) |
| **Honey 1** | 106.71 | 114.78 | 729.33 | 722.96 |
| **Honey 2** | 37.44 | 33.11 | 459.53 | 428.56 |
| **Honey 3** | 120.90 | 122.95 | 727.39 | 752.40 |
| **Honey 4** | 46.85 | 49.44 | 485.84 | 487.44 |
| **Honey 5** | 30.16 | 22.22 | 395.83 | 389.30 |
| **Honey 6** | 53.91 | 57.61 | 470.66 | 516.88 |
| **Honey 7** | 93.68 | 90.28 | 615.07 | 634.64 |
| **Honey 8** | 35.72 | 38.55 | 447.48 | 448.18 |

1. **Conclusions**

Chemical sensors, or chemosensors, have great potential in the field of *in-situ*, fast, and low-cost analysis. Among chemical sensors, polymeric sensors have advantages of lack of migration of the sensor subunits, manageability, and possibility of working in solid-state. We have studied a sensor with diazonium moieties pendant to the main acrylic chains that can be used as a colorimetric chemosensor for the quantification of **TPC** and the determination of **AOX** on honey samples. The color of the sensors changes according to the samples' polyphenols concentration. This sensor allows us to determine the **TPC** and the **AOX** of different honey samples in a single measurement, in 2h (see **ESI S6**) , by only taking a photograph to the sensory colorimetric films after immersion in honey samples, avoiding high-cost methodologies that require expensive equipment, trained personnel and long analysis time. This sensor has great potential in the quality control of honey samples reducing costs derived from this type of analysis, deeply diminishing the environmental impact of the measurements and greatly speeding up the analysis time.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

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