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Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer

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ABSTRACT

Glucose oxidase (GOX) and catalase (CAT) regulate the amount of H2O2 in honey, by generating or consuming it, so they are related to the antibacterial and antioxidant activity of honey. However, their activities are hardly analysed, since the process requires a previous dialysis that is non-selective, very time-consuming (>24 h), eco-unfriendly (>6L of buffer) and expensive. This research shows the design and performance of a material that selectively removes the actual interferents. The film-shaped-polymer is immersed for 90 within a honey solution (12.5 mL of buffer), where it interacts exclusively with 1,2-dihydroxybenzenes, which we proved to be the real interferents (the material contains motifs derived from phenylboronic acid to interact with 1,2-diols). Polymeric chains favour condensation to occur exclusively with 1,2-dihydroxybenzenes, excluding monosaccharides. The interferents' removal using our designed polymer is selective, low cost (1.42€ per test), rapid and eco-friendly (saves 6L of buffer and 20.5 h of experimental workout per sample).

1. Introduction

Polyphenols, reducing sugars, minerals, organic acids, vitamins, amino acids, substances responsible for aroma and flavours, or enzymes, are just some examples of the wide variety of compounds present in honey, a foodstuff produced by bees (White & Doner, 1980).

Behind China, the European Union is the second-largest producer of this natural sweetener, with around 600,000 beekeepers and 17 million hives producing 250,000 tons of honey each year. The consumption of this food is so high in Europe that around additional 200,000 tons must be imported annually (Duch Guillot, 2018).

Considering the conspicuous business, one of the priority objectives of the European Union is the quality control of the products produced inside and outside Europe through the characterization of the most relevant properties of honey, their antioxidant and antibacterial activities. The first is mainly due to the presence of polyphenols, while many authors consider that the most relevant antibacterial agent present in honey is hydrogen peroxide (H_2O_2). The concentration of this substance in honey is determined by the activity of two enzymes: glucose oxidase (GOX) and catalase (CAT). GOX generates H_2O_2 in the glucose oxidation process, whereas CAT breaks it down, generating water and oxygen. Additionally, the activity of these enzymes decreases with the storage time. Therefore, the measurement of the enzyme activity is one of honey's most important quality parameters (Huidobro, Sánchez, Muniategui, & Sancho, 2005; Schepartz & Subers, 1966).

In the last decades, the determination of GOX and CAT activities in honey samples has been carried out by different methods, most of which are based on H_2O_2 quantification before and after an incubation period. Dold & Witzenhausen, 1955, have quantified GOX activity by a microbiological procedure using *Staphylococcus aureus*. The method was precise enough but tedious to be used for routine determinations. Several authors quantified H_2O_2 by titration (Kiermieier & Köberlein, 1954; Salashinski & Bazhenova, 1979). However, these methods were not suitable for the determination of GOX and CAT activities because

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titration reagents involved secondary reactions (Griebel & Heß, 1940; Schepartz & Subers, 1966). So far, the most used method to determine both GOX and CAT activities is based on spectrophotometric measurements of H_2O_2. Some assays determined GOX activity directly on honey solutions (Flanjak, Strelec, Kenjerić, & Primorac, 2016; Sahin, Kolayli, & Beykaya, 2020; White, Subers, & Schepartz, 1963), but honey interferences made these procedures unreliable for analytical purposes (Kausaite-Minkstimiene, Kaminskas, Popov, Ramanavicius, & Ramanaviciene, 2021; Sánchez Castro, 2000; Schepartz & Subers, 1964). To properly determine honey's GOX and CAT activities spectrophotometrically, a purification step by dialysis proved to be mandatory in order to remove all interferents (Schepartz & Subers, 1966). Despite being used for decades, dialysis is a very tedious process that takes 24 h, consuming 6 L of phosphate buffer at pH 7 for each honey sample. It is based on the use of commercial dialysis membranes, being a very abrupt procedure that allows different compounds to pass through the membrane, such as sugars (fructose, glucose, maltose, among others), polyphenols and other honey constituents. Nevertheless, neither the real interferent substances nor the actual necessity for all those compounds removal have been researched so far.

The aim of this work is to study and describe the real interferents for the determination of GOX and CAT activities in honey and, eventually, to develop a simple and easy alternative and good value-for-money method to remove all actual interferences for the reliable spectrophotometric measurement of both enzymatic activities in honey. The solid-phase extraction has already been used for honey purification purposes (Azzouz & Ballesteros, 2015; Galarini, Saluti, Giusepponi, Rossi, & Moretti, 2015; Surma, Wiczkowski, Cieślik, & Zieliński, 2015), so the studied assay is based on the design of a polymeric material for the highly specific removal of those interferents via solid-phase extraction, as graphically depicted in Fig. 1. The proposed new strategy is carried out in 90 min and using only 25 mL of phosphate buffer (pH 7), leading to an improvement of 94 % and 99.6 % in the experimental time and buffer volume, respectively. Furthermore, the polymeric material is made with 100 % commercially available monomers, which favours its future industrial scaling and its use in the food industry and research.

2. Materials and methods

2.1. Honey samples

This study was carried out with 29 representative kinds of honey 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 activities of this country (Ministerio de Agricultura, 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 et al., 2004), there was 2 lavender (Lamiaceae type *Lavandula* sp.) honeys (samples 1 and 12), 3 forest honeys (samples 2, 10 and 22), 12 ling heather (Ericaceae type *Calluna vulgaris*) honeys (samples 3, 5, 6, 8, 9, 14, 15, 16, 20, 21 and 24), 7 multifloral honeys (samples 13, 23, 27, 28 and 29), 1 centaury flower (*Centaurea cyanus*) honey (sample 11), 1 holly (*Ilex aquifolium*) honey and 3 honeydew honeys (samples 19, 25 and 26). 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., Compositae type *Helianthus annuus* L. and Ericaceae type *Erica* spp. All honey samples were fresh and tested as soon as they were received.

2.2. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**) (Aldrich, 99 %), *N,N*-dimethylaminoethyl metacrylate (**NNDA**) (Aldrich, 98 %), 2-hydroxyethyl acrylate (**2HEA**), (Aldrich, 96 %), ethylene glycol dimethacrylate (**E**) (Aldrich, 98 %), hydrochloric acid (VWR, 37 %), methanol (Aldrich, 99.8 %), 4-vinylphenylboronic acid (**BOR**) (TCI, 95 %), sodium dihydrogen phosphate (Merck, 99.99 %), di-sodium hydrogen phosphate anhydrous (Merck, 99.99 %), potassium phosphate monobasic (Aldrich, ≥99 %), sodium phosphate dibasic dihydrate (Panreac, ≥99 %), ammonium metavanadate (Aldrich, ≥99 %), sulfuric acid (VWR, 95 %), *o*-dianisidine dihydrochloride (Sigma, >99 %), hydrogen peroxide (Aldrich, ≥30 %), catechol (Aldrich, 99 %), resorcinol (Merck, 99 %), hydroquinone (Aldrich, 99 %), quercetin hydrate (Aldrich, 95 %), D-(+)-Glucose (Panreac, 99.99 %), D-(−)-Fructose (Aldrich, 99 %), D-(+)-Maltose (Sigma, 97.5 %), sodium permanganate monohydrate (Aldrich, ≥95 %), peroxidase from horseradish (Sigma Aldrich, EC 1.11.1.7), dialysis membranes (Aldrich, D6191), *d*imethylsulfoxide-d_6 (VWR, 99.9 %), catalase from beef liver (EC 1.11.1.6) from Roche

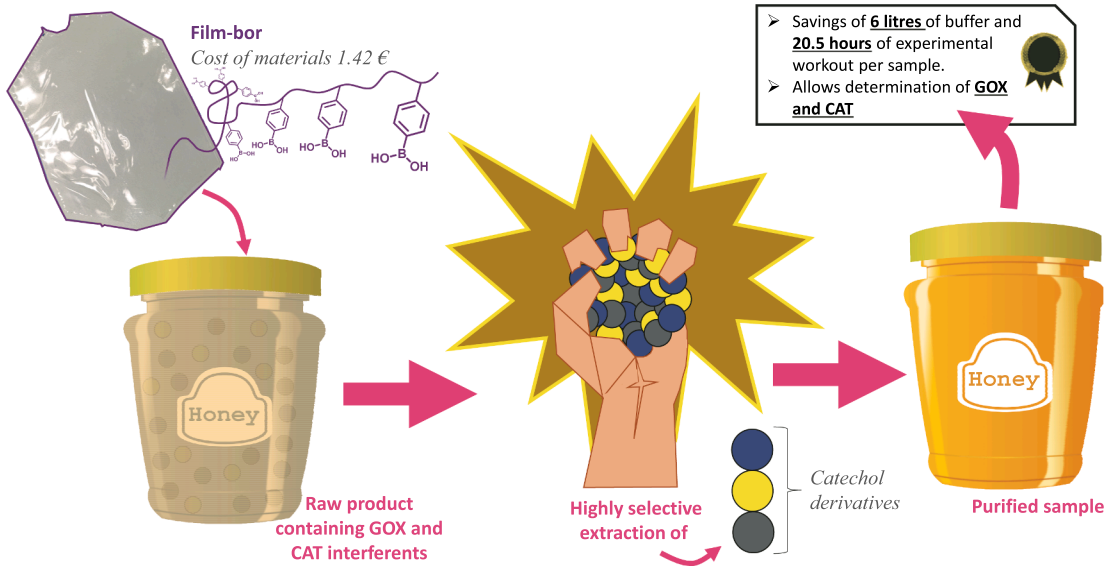

Fig. 1. Graphical representation of the proposed methodology for honey purification and enzyme activity determination (GOX and CAT).

Diagnostics (Mannheim, Germany), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) from Sigma Aldrich. Azo-bis-isobutyronitrile (AIBN, Aldrich, 99 %) was recrystallized twice from methanol.

2.3. Preparation of the polymeric film and linear polymer containing phenylboronic acid groups (*Film-bor* and *Linear-bor*)

The polymeric polyacrylic film **Film-bor** was prepared by bulk radical polymerization of four commercial monomers: *N,N*-dimethylaminoethyl methacrylate (**NNDA**), 2-hydroxyethyl acrylate (**2HEA**), methyl methacrylate (**MMA**) and 4-vinylphenylboronic acid (**BOR**) in a molar feed ratio of 42.5/42.5/10/5 (**NNDA/2HEA/MMA/BOR**) using 1 % weight of AIBN as radical thermal initiator, and 5 % mol of ethylene glycol dimethacrylate (**E**) as the crosslinking agent. This formulation was optimized for working with honey samples in previous works (González-Ceballos et al., 2021). The polymerization was carried out overnight at 60 °C in a mould comprised between two silanised glasses (100 μm thick), in an oxygen-free atmosphere. **Film-bor** was removed from the mould, washed three times with methanol, and dipped in HCl (4 %). The treatment with HCl protonates the tertiary amine group of **NNDA**, improving the water swelling percentage of the material. Finally, the **Film-bor** was washed several times with water until the pH of the washing water was 7. Complete characterization of the polymer can be found in Supplementary Material (SM)**, Section S1**. Fig. 2**a** shows the chemical structure of **Film-bor** and a real image of the prepared material.

Linear-bor polymer was prepared by radical co-polymerization of the same commercially available monomers as **Film-bor**, and in the same molar ratio, i.e., **NNDA/2HEA/MMA/BOR** (42.5/42.5/10/5). First, 15.9 mmol of NNDA (2.5 g), 15.9 mmol of 2HEA (1.85 g), 3.7 mmol of MMA (374 mg), and 1.87 mmol of BOR (277 mg) were dissolved in DMF (18.7 mL), and the solution was added to a round-bottom pressure flask. Subsequently, radical thermal initiator AIBN (307 mg, 1.87 mmol) was added, the solution was sonicated for 10 min, and heated overnight at 60 °C, under a nitrogen atmosphere, and without stirring. The solution was then dropwise added to diethyl ether (150 mL) with magnetic stirring, yielding the desired product as a yellowish precipitate. Finally, polymers were purified in a Soxhlet apparatus with diethyl ether as washing solvent to eliminate DMF traces. Yield ≈ 65 %. Complete polymer characterization can be found in the electronic supporting information (**SM-Section S1**). Fig. 2**b** shows the chemical structure of **Linear-bor**, and a real image of the prepared polymer.

2.4. Instrumentation

Inductively coupled plasma mass spectrometry (ICP-MS) measurements were recorded on an Agilent 7500 ICP-MS spectrometer (Agilent, Santa Clara, USA). ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra (Avance III HD spectrometer, Bruker Corporation, Billerica, Massachusetts, USA) were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C using deuterated solvents such as dimethyl sulfoxide (DMSO-d_6) at 25 °C.

The weight percentage of water taken up by the films upon soaking in pure water at 20 °C until reaching equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film (ω_d) and its water-swelled weight (ω_s) using the following expression: $WSP = 100 \times [(\omega_s-\omega_d)/\omega_d]$. The powder X-ray diffraction (PXRD) patterns were obtained using a diffractometer (D8 Discover Davinci design, Bruker Corporation, Billerica, Massachusetts, USA) operating at 40 kV, using Cu (Kα) as the radiation source, a scan step size of 0.02°, and a scan step time of 2 s.

The polymers were thermally characterized by using thermogravimetric analysis (Q50 TGA analyzer, TA Instruments, New Castle, DE, USA) with 10–15 mg of sample under synthetic air and nitrogen atmosphere at 10 °C·min^{-1}; differential scanning calorimetry, with 10–15 mg of the sample under a nitrogen atmosphere at a heating rate of 10 °C min^{-1} (Q200 DSC analyzer, TA Instruments, New Castle, DE, USA); and tensile properties analysis, with 5 × 9.44 × 0.103 mm samples tested at 5 mm min^{-1} (EZ Test Compact Table-Top Universal Tester, Shimadzu Kyoto, Japan). Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) with an ATR-PRO410-S single reflection accessory.

Isothermal titration calorimetry (ITC) measurements were performed using a microcalorimeter (VP-ITC MicroCal Inc., Malvern, UK) equipped with two cells, one cell for sample (**Linear-bor**), at a final concentration of phenylboronic acid groups of 0.61 mM, and another one for reference, with volumes of 1.436 mL. The titrant syringe was filled with 280 μL solution (9 μM) of the tested compounds (catechol, resorcinol, hydroquinone, fructose, glucose, and maltose), which was step-by-step added to the sample cell (20 μL aliquots were added every 5 min). All the solutions were degassed for 10 min at 25.0 ± 0.1 °C in a vacuum pump to avoid blistering in the syringe or in the calorimetric cells during the experiment. Calorimetric measurements were performed at 25.000 ± 0.001 °C, with a constant stirring at 307 rpm in the sample cell.

The catechol concentration in aqueous solutions used in the permeation, kinetic and sorption isotherm analysis was determined via UV–vis spectrophotometer measurements (UV-2600*i*, Shimadzu, Germany). Calibration line was performed for catechol in the analytical range 0–50

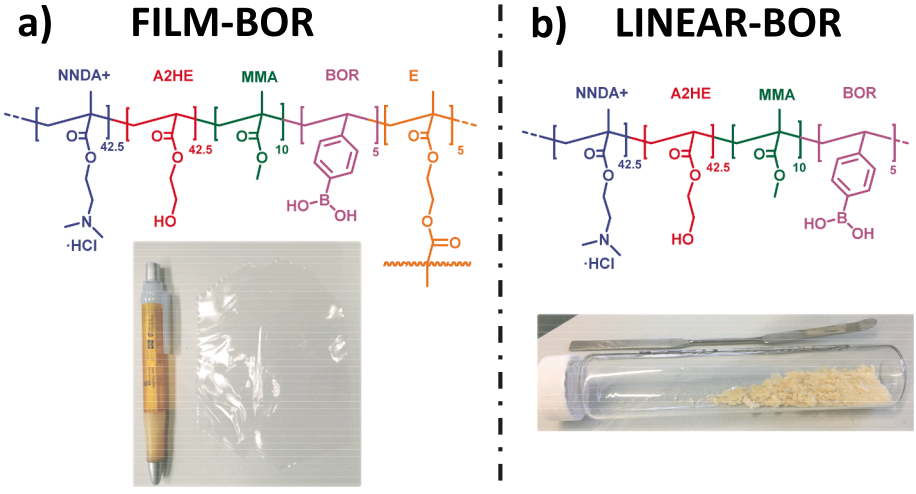

Fig. 2. Chemical structures and real images of **a**) the membrane **Film-bor**, and **b**) the water-soluble linear polymer **Linear-bor**.

mg L^{-1} by using the absorbance at $\lambda^{max} = 275$ nm; a molar absorption coefficient equal to 48.6 L mol^{-1} cm^{-1} was obtained. The analysis was performed in duplicate, and the solutions were prepared in Milli-Q ultrapure water.

Enzyme interferents assays were performed using a Synergy HT microplate reader (BioTek®, Winooski, Vermont, USA), measuring absorbance at 452 nm. The concentration of CAT, GOX, H_2O_2, and the compounds used as interferents was determined using a Hitachi U-3900 UV–vis spectrophotometer (Tokyo, Japan).

The enzymatic (GOX and CAT) activities were measured using a CARY 400 Bio UV–Visible Spectrophotometer, measuring the absorbance, for both analyses, at 400 nm.

2.5. Methods

2.5.1. Honey purification for the determination of the activity of GOX and CAT using Sigma Aldrich dialysis membranes D6191

The membranes had to be conditioned first, following the manufacturer's instructions. Therefore, the membrane was immersed in a beaker containing boiling water for 2 min, and the process was repeated for the same time in another beaker, avoiding the membrane cooling down between the first and second washes. Finally, the membrane was immersed in water at room temperature.

7.5 g of honey was dissolved in phosphate buffer 0.015 M at pH 7 (4 mL) in a beaker. The lower end of the already conditioned membrane was closed with a clamp, and the solution was placed inside it using a funnel. The beaker was washed several times with buffer, using 8 additional mL, and finally, all portions were homogenized inside the membrane. After closing the upper end of the membrane with another clamp, it was dipped in a beaker containing 3 L of phosphate buffer 0.015 M and the system was kept at 4 °C. The buffer was renewed (3 L) after 10 h, and the system was carefully homogenized every 6 h. After 22 h, the membrane was removed from the beaker and washed with the buffer solution. The content of the membrane was transferred to a 50 mL volumetric flask, and the same buffer was used to clean the interior of the membrane and make up to the mark.

2.5.2. Honey purification for the determination of the activity of GOX and CAT using **Film-bor**

7.5 g of honey were homogenized with 12.5 mL of 0.015 M phosphate buffer and placed in a beaker with a film blanket. The system was gently stirred for 90 min at room temperature. Finally, the membrane was removed from the beaker, and the solution was transferred to a 50 mL volumetric flask and made up to the mark using the same buffer.

2.5.3. Fundamentals on the determination of CAT activity in honey samples

The quantification of the CAT activity is based on the reaction between CAT and an excess of H_2O_2 (substrate); the non-reacted amount of H_2O_2 is then made to react with *o*-dianisidine and peroxidase, and the obtained coloured product is quantified by spectrophotometry, at 400 nm. Purified honey samples with methods depicted in sections 2.5.1 and 2.5.2 were tested following the detailed experimental procedure published by Huidobro et al. in 2005, with modifications. More details about the experimental procedure can be found in **SM-Section S2**.

2.5.4. Determination of GOX activity in honey samples

Contrarily to CAT, H_2O_2 is the reaction product (not the substrate) produced when GOX reacts with glucose. Therefore, the method for GOX activity determination is based on the addition of known amounts of glucose to the honey sample and the quantification of the generated H_2O_2. Similarly to CAT, the quantification of GOX activity is based on measuring the absorbance at 400 nm of the coloured product formed by reacting *o*-dianisidine and peroxidase with H_2O_2. Purified samples with methods depicted in sections 2.5.1 and 2.5.2 were tested based on the experimental procedure reported elsewhere by Schepartz & Subers in 1966. More details about the experimental procedure can be found in **SM-Section S3**.

2.5.5. Interference study

The study was carried out with catechol, resorcinol, hydroquinone, D-fructose, D-glucose, and maltose as possible interferents. Since catechol is a peroxidase substrate (García-Moreno, Moreno-Conesa, Rodríguez-López, García-Cánovas, & Varón, 1999; Huidobro et al., 2005), this study could not be performed using the method described in sections 2.5.3. and 2.5.4. For this reason, we have used a modified version of the method described by Hadwan & Ali, in 2018. Inhibitory capacity of catechol, resorcinol, hydroquinone, glucose, fructose, and maltose was tested *in vitro* against commercial CAT and GOX using 96-well microplates. Basically, 10, 50, and 150 mg L^{-1} of tested compounds were preincubated for 10 min with 20 ng of CAT or 1 h with 4 μg of GOX in a 50 mM phosphate buffer at pH 7. Subsequently, substrates were added at a final concentration of 4 mM (H_2O_2) or 80 mM (D-glucose) and incubated for 2 or 20 min at 37 °C for CAT or GOX activity, respectively. Thereafter, the presence of H_2O_2 in the reaction was detected by the addition of ammonium metavanadate at a final concentration of 4 mM and 10 min of incubation at 28 °C. Then, the absorbance was measured at 452 nm. System was calibrated with known amounts of H_2O_2 in a standard reaction mixture. Inhibitory activity of the analysed compounds was expressed as percentage of enzymatic activity relative to that in the absence of the tested compound. All assays were conducted by triplicate, and blanks were used to account for spontaneous hydrolysis of the substrates and unspecific reaction of the interferents with the rest of the reaction compounds.

2.5.6. Permeation, kinetic and isotherm study of **Film-bor**

The permeation study was carried out with a system of horizontal communicating vessels (Valente, Polishchuk, Lobo, & Burrows, 2000). The studied material (**Film-bor**) was placed as a barrier in the channel between the two vessels, whose permeation area is 2 cm^2. Milli-Q ultrapure water was placed in vessel "A", and an aqueous solution of catechol at 5000 mg L^{-1} was placed in vessel "B". The permeation process was studied by measuring the absorbance at 275 nm of the solution in vessel "A" over time.

Sorption analyses were performed at 25 °C, using a solid/liquid ratio of 50 mg of **Film-bor** (dried at 60 °C for 2 h) per mL of solution. In detail, for kinetic sorption analysis, 0.15 g was dipped in 3 mL of an aqueous solution of catechol (15 mg L^{-1}) within 0–35 min. The mechanism was studied by fitting pseudo-first order (PFO) and pseudo-second order (PSO) models to the experimental data. The non-linear equations are:

$$q_t = q_e\left(1 - e^{-k_1 t}\right) \quad (1)$$

$$q_t = \frac{k_2 q_e^2 t}{1 + k_2 q_e t} \quad (2)$$

where q_t (mg g^{-1}) is the amount of sorbate at a defined interval of times (min) that at equilibrium state is defined as (mg g^{-1}). k_1 (min^{-1}) and k_2 (g mg^{-1} min^{-1}) are the rate constants for PFO and PSO, respectively (William Kajjumba, Emik, Öngen, Kurtulus Özcan, & Aydın, 2019).

The goodness of fitting of the two models is compared by the Akaike information criterion (AIC) (eq. 3)

$$AIC = Nln\left(\frac{RSS}{N}\right) + 2k + \frac{2k(k+1)}{N-k-1} for \frac{N}{k} < 40 \quad (3)$$

where N is the number of the experimental points, RSS the residual sum of squares and k the number of fitted parameters (Kingdom & Prins, 2016).

The isotherm analysis was performed using 0.1 g of **Film-bor** in 2 mL of aqueous catechol solutions at concentrations ranging from 0 to 500 mg L^{-1}. The batches were shaken in an incubator (ZWY-100H, Labwit) at constant temperature and 120 rpm. The Langmuir isotherm model was fitted to the experimental data to better understand the sorption

process. The mathematical expression is described by equation (4).

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (4)$$

where q_m (mg g^{-1}) is the maximum sorption capacity per unit weight of sorbent, C_e (mg L^{-1}) is the concentration of analyte at equilibrium state, and K_L (L mg^{-1}) is the Langmuir constant.

The amount of sorbate per gram of sorbent at equilibrium state, q_e, and the removal efficiency of the sorbent, *RE%*, were determined by the following equations:

$$q_e = \frac{C_0 - C_e}{m} \times V \quad (5)$$

$$RE\% = \frac{C_0 - C_e}{C_0} \quad (6)$$

where C_0 (mg L^{-1}) is the initial concentration of catechol, m (g) is the mass of the sorbent, and V (L) is the volume of the solution.

2.5.7. Statistical analyses

The statistical analysis was performed using GraphPad Prism v8. Normality and homoscedasticity of the data were first analysed. When the data fulfilled both assumptions, Two-way ANOVA was conducted to analyze the enzyme activity inhibition with several compounds at different concentrations, and Tukey's multiple comparisons test ($p < 0.05$) was used. In addition, Two-way ANOVA was used to compare CAT and GOX activities in honey samples, comparing values within each row followed by Uncorrected Fisher's LSD test ($p < 0.05$).

3. Results and discussion

3.1. Interference study

The main purpose of this work was based on the study of the real interferents for the determination of honey's GOX and CAT activities. Many authors considered that the interferents were mainly reducing sugars (85 % of honeýs total composition) (Sánchez Castro, 2000; Schepartz & Subers, 1964). On the other hand, polyphenols such as catechol and its derivatives were identified in some publications as CAT interferents (Krych & Gebicka, 2013). Furthermore, some studies pointed out that far from being interferents, some reducing sugars as glucose increased the activity of CAT (Akbayirli & Akyilmaz, 2008). However, this matter is far from being clarified, due to the existing contradictory positions. Thus, one of our goals is to contribute to this clarification.

GOX and CAT activity assays have been performed with some of the major honey compounds, such as sugars and polyphenols, which were thought as the potential interferents of these enzymes. On the one hand, the decision for sugars was straightforward, as D-fructose, D-glucose, and maltose are the main honey carbohydrates. On the other hand, the decision on polyphenols was not so clear since there are many different polyphenols in honey. Thus, we tackled this issue from the chemical point of view, classifying polyphenols as 1,2-1,3- and 1,4-dihydroxybenzene derivatives. In this way, and always from a chemical viewpoint, all polyphenols were represented by catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) structures.

As shown in Fig. 3, the 3 different types of dihydroxybenzenes (1,2-, 1,3-, and 1,4-) were clear interferents of CAT activity, unlike the 3 selected reducing sugars. On the other hand, all the interferents studied seemed to have the same inhibition effect on GOX activity, both polyphenols and reducing sugars. However, this inhibition was considerably lower, under 27 % in all cases. Furthermore, glucose is the substrate in the tests with GOX at a concentration of 80 mM (14,412 ppm). As some authors have detailed (Kausaite-Minkstimiene et al., 2021), glucose can be an inhibitor of glucose oxidase at high concentrations, so we interpret that the inhibition we see in this assay is due to this fact. These results were the basis for the design of the polymeric material.

3.2. Design of the polymer structure for the extraction of polyphenols from honey samples

As shown by the interference study, polyphenols are the real interferents for determining GOX and CAT activities in honey. The phenolic profile of honey is highly variable and depends largely on its botanical origin as well as on other factors such as geographical factors, among others (Yayinie et al., 2022). However, when talking about honey, we can assume that the vast majority of polyphenols contain 1,2-dihydroxybenzenes in their structures, mainly phenolic acids (gallic acid, caffeic acid and chlorogenic acid), and flavonoids (catechin, quercetin and luteolin) as studied by several authors and as depicted in Fig. 4**a** (Cheung, Meenu, Yu, & Xu, 2019; Ciulu et al., 2016; Khalil, Alam, Moniruzzaman, Sulaiman, & Gan, 2011; Pandey & Rizvi, 2009). Thus, we decided to synthesize a material able to extract these compounds in a very selective and effective manner. In this way, the honey sample would be as similar as possible to its natural state, but without these

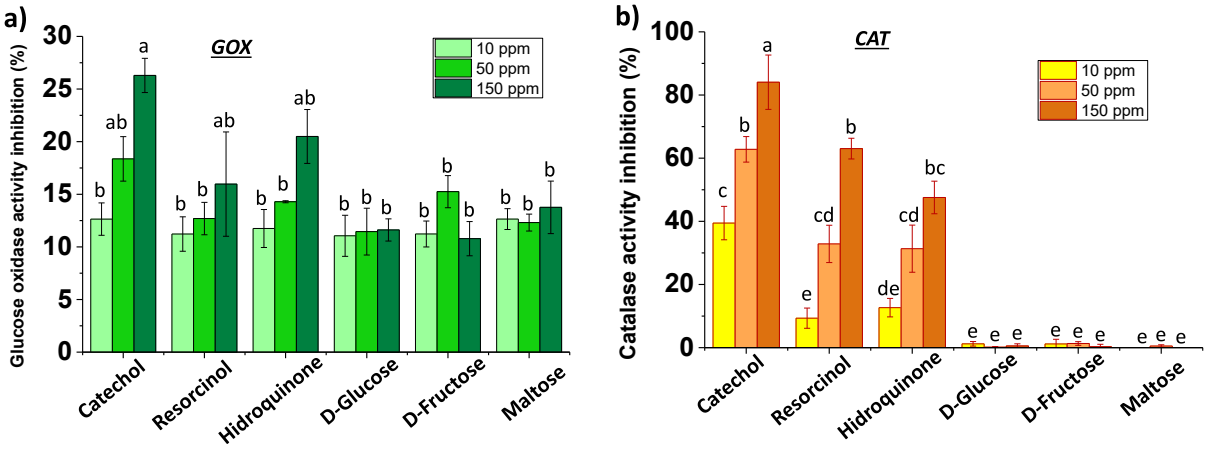

Fig. 3. Inhibitory ability of catechol, resorcinol, hydroquinone, glucose, fructose and maltose against commercial CAT and GOX: a) percentage of GOX activity inhibition by catechol, resorcinol, hydroquinone, glucose, fructose and maltose at different concentrations (10, 50, 150 mg L^{-1}); and b) percentage of CAT activity inhibition by catechol, resorcinol, hydroquinone, glucose, fructose and maltose at different concentrations (10, 50, 150 mg L^{-1}). Data are mean ± SE of three independent replicates. Different letters indicate significant differences within each commercial enzyme activity inhibition ($p < 0.05$, two-way ANOVA followed by Tukey's multiple comparisons test).

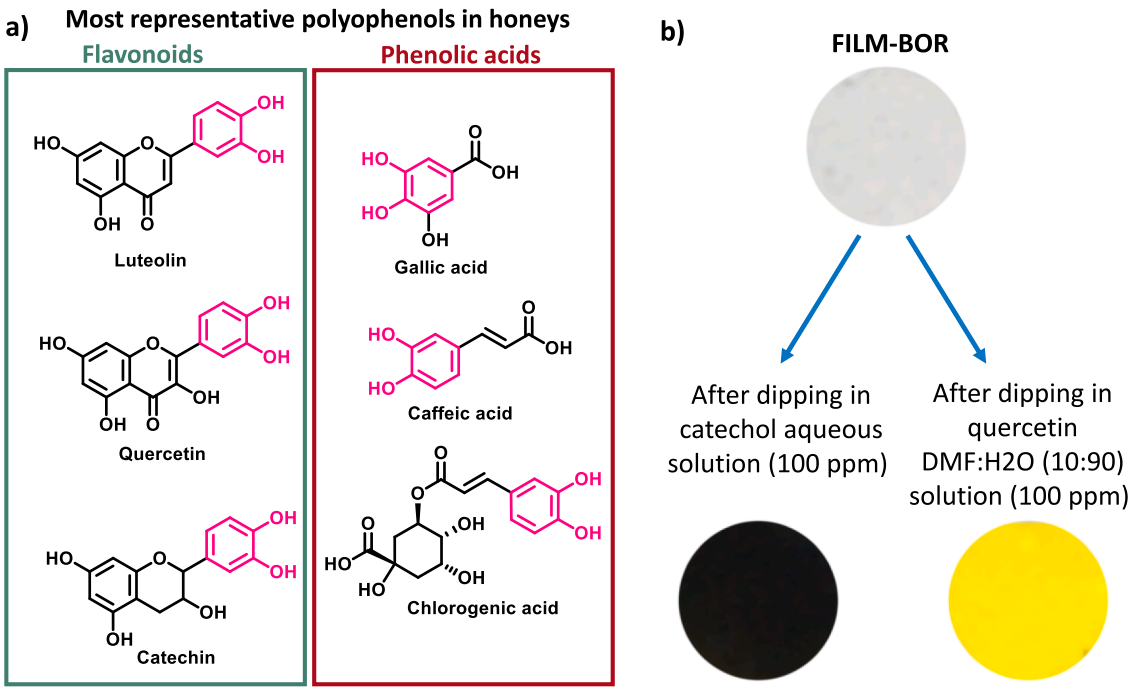

Fig. 4. Interaction between **Film-bor** and 1,2-dihydroxybenzenes: a) Most representative polyphenols in honey, containing 1,2-dihydroxybenzene derivatives; b) qualitative experiment with catechol and quercetin. A 10 mm diameter disc of **Film-bor** was dipped in 100 ppm aqueous solutions of catechol and quercetin. For the latter, 10 % of dimethylformamide had to be added to improve solubility.

interferents.

Our first hypothesis was to prepare a polymeric material with phenylboronic pendant groups since the formation of boronic esters with catechol derivatives (Hagihara, Tanaka, & Matile, 2008; Suzuki et al., 2020) and polyphenols in general (Hall, 2005) had been widely studied. However, phenylboronic acid derivatives were also studied as sensors for recognizing sugars (Matsumoto, Sato, Kataoka, & Miyahara, 2009), and this could be somehow negative. Therefore, our second hypothesis was to modulate the reactivity of the polymeric material to react only with the 1,2-dihydroxybenzene derivatives. This fact could only be achieved with an appropriate design of the rest of the monomers used to prepare the material. In other words, the polymers should not only include receptors based on phenylboronic groups (5 mol%), but the rest of the monomers (95 mol%) should also be selected *ad hoc* for the proposed objective, i.e., to extract GOX and CAT activity interferents from honey.

After several tests with different formulations containing 5 mol% of phenylboronic pendant groups (see **SM-Section S4**), we concluded that the best one was based on **NNDA**, **2HEA**, **MMA** and **BOR**. Additionally, the material was conditioned in an acidic medium (HCl, 4 %) to protonate the **NNDÁ**s tertiary amine groups and thus promoting the formation of boronic esters (Matsumoto et al., 2009). As a remark, we qualitatively carried out this preliminary study with catechol and quercetin, two of the most abundant 1,2-dihydroxybenzene derivatives in honey. As shown in Fig. 4**b, a** drastic material colour change is observed in solutions of catechol (black) and quercetin (yellow). For the rest of the study, we only worked with catechol as the model for 1,2-dihydroxybenzene derivatives due to the poor water-solubility of quercetin.

Finally, it must be highlighted that the material cost to carry out one test is only 1.42 €, so the methodology is technically and economically viable.

3.3. Isothermal titration calorimetry experiments (ITC).

The key part of the ITC experiments was the choice of the concentrations of **Linear-bor** and the species studied. Since the greatest interference for the measurement of enzymatic activity was observed with catechol, these concentrations were adjusted using this compound as a model.

The concentration of catechol in honey can vary from 1.1 to 87.6 mg L^{-1} depending on the type of honey (Da Silva, Da Silva, Camara, Queiroz, Magnani, Novais, & De Souza, 2013), so we set 50 mg L^{-1} of catechol, as well as other species, as the starting point for this study. It is worth noticing that the concentration of sugars in honey is much higher; however, we choose to work with the same concentrations for all carbohydrates to allow a reliable comparison and conclusions, as well.

ITC measurements are a powerful technique to provide information on the thermodynamic parameters, including the equilibrium constant (K_{eq}), the enthalpy (ΔH) and the entropy (ΔS) of interaction.

Regarding equilibrium constants, phenylboronic acid derivatives generally suffer condensation reactions with 1,2- and 1,3- diols, both in acid and alkaline media (Davis, 2001); however, as a general approach, equilibrium constants increase by increasing the pH (Springsteen & Wang, 2002). Specifically, with sugars, the reaction depends on the conformation of the monosaccharides and, therefore, on the spatial position of the –OH groups. The most favourable conformation for the formation of the phenylboronic ester was the *syn*-periplanar provided by the furanose structure (Elshaarani et al., 2018). Consequently, the equilibrium constants reported for fructose were much higher than for glucose, since the α and β tautomers of fructofuranose have abundances of 28 % in honey samples (Mazzoni, Bradesi, Tomi, & Casanova, 1997), while the abundance of the α and β tautomers of glucofuranose only reached up to 0.3–1.3 % in aqueous solutions (Maple & Allerhand, 1987) and 0 % in honey samples (Mazzoni et al., 1997).

At pH 7.4, the equilibrium constants found in the literature for interaction between boronic acid and D-fructose, D-glucose, and maltose are 160, 4.5 and 2.5 M^{-1}, respectively. However, the K_{eq} for catechol is significantly higher, 830 M^{-1} (Springsteen & Wang, 2002); this value was justified due to the high rigidity of 1,2-dihydroxybenzene derivatives, which facilitates the boronic ester formation.

Therefore, based on previously reported data, it is expected that our

material might interact mainly with catechol and fructose and no (or less intense) interaction should occur for glucose, maltose, resorcinol and hydroquinone. Fig. 5**a** shows the heat per injection for solutions of **Linear-bor** when adding selected carbohydrates or polyphenols. ITC detects only the reaction with catechol. The corresponding equilibrium constant for catechol containing solution is 1110 ± 104 M^{-1} (individual graphs for each tested compound can be found in **SM-Section S5**). It can also be concluded that the interaction is exothermic (ΔH = 13.7 ± 0.6 kJ mol^{-1}) and enthalpy-driven (TΔS = 3.7 kJ mol^{-1}), which suggests a strong catechol-**Linear-bor** interaction, and the role of solvent can be essentially neglected.

However, it must be stressed that the selective behaviour towards catechol was considerably better than those initially expected and can be attributed to the environment generated by the polymer chains, which definitely favoured interaction with catechol, hindering interaction with sugars. Without the environment generated by polymer chains, the interaction with monosaccharides, especially with fructose, cannot be measured, essentially due to the low solubility of the monomer in water (Lorand & Edwards, 1959; Springsteen & Wang, 2002).

3.4. Permeation, kinetic, and isotherm study of *Film-bor*

In line with ITC experiments using **Linear-bor**, the permeation study showed a chemical interaction between **Film-bor** and catechol. As shown in Fig. 5**b**, the time needed to reach a steady-state flux, which corresponds to the time-lag (q = 2.0 h), is significantly high, indicating that before permeating, the main process is an interaction between catechol and the **Linear-bor**, in which catechol is retained in the membrane, probably through a chemical condensation reaction.

Considering the **Film-bor** membrane thickness (L = 100 μm), the permeation area (A = 2 cm^2), the cell volume (V = 200 mL), and the concentration of catechol in the donor cell (C_{cat}), the diffusion (D) and permeation (P) coefficients can be calculated by the following expressions:

$$D = L^2/6\theta \tag{7}$$

$$P = (V/A) \times m \times (L/C_{cat}) \tag{8}$$

where m is the slope of the variation of concentration of catechol that permeates the membrane as a function of time, at the steady-state condition (see red dashed line in Fig. 5**b**). Thus, we obtained a diffusion coefficient value of 2.2 × 10^{-9} cm^2 s^{-1}, and a permeation coefficient value of 2.8 × 10^{-7} cm^2 s^{-1}. As a negative control, we carried out the same experiment with resorcinol, obtaining very similar results for P (5.25 × 10^{-7} cm^2 s^{-1}), but one order of magnitude higher value for D (1.72 × 10^{-8} cm^2 s^{-1}). These results lead to a partition coefficient (K = P/D) for catechol equal to 128, 420 % higher than that found for resorcinol (30). These values show a high affinity of catechol towards **Film-bor** membrane, in close agreement with the ITC results.

The kinetic sorption of catechol (15 mg L^{-1}) into **Film-bor** (**SM-Section S6,** Figure S6**a**) indicates that the equilibrium is reached in about 10 min. Considering that the sorption thickness is L/2, compared with that for the permeation experiments, such a time matches with the first step of the permeation analysis, i.e., at unsteady-state conditions (see Fig. 5**b**). It can be hypothesized that such behaviour can be justified by the saturation of the active sites inside the membrane – a prerequisite for achieving the steady-state flux. Surprisingly, the overall sorption kinetics data are better fitted by the PFO kinetic model (**SM-Section S6,** Table S1). This suggests a diffusion-controlled process once PFO model is comparable to a mass action rate for sorption seen as a transfer process (Vareda, Valente, & Durães, 2016). For a deeper assessment on these results, the weight of data at short-range times (in this case, for $q_t/q_e <$ 0.85) for the overall fitting was evaluated by using the Boyd equation (William Kajjumba, Emik, Öngen, Kurtulus Özcan, & Aydın, 2018), $B_t = mt + C$, where B_t is defined as.

$$B_t = 2\pi - \frac{\pi^2 q_t}{q_e}/3 - 2\pi\sqrt{\left(1 - \frac{\pi q_t}{q_e}/3\right)} \tag{9}$$

m is the slope, and C is a constant which defines the rate-limiting step. By fitting the Boyd equation to the experimental sorption data shown in Figure S6**a**, we obtained C = -0.133 (0.006), with a determination coefficient equal to 0.9939. This value clearly indicates that at short-range times a time lag governs the process, whilst for long-range times the process is mainly diffusional, in close agreement with the conclusions obtained above and the sorption isotherms as discussed further below.

Concerning the sorption isotherm, in **SM-Section S6,** Figure S6**b**, the Langmuir model perfectly fitted the experimental data indicating a homogeneous sorption system on active sites of the polymer. Once again, the results show that the boronic acid groups might act as active sites at the same energy for interacting with catechol. The favourable sorption process was also indicated by Webber-Chakkravorti constant, 0 < R_L < 1 (**SM-Section S6** Table S1). Finally, we must highlight that the **Film-bor** showed a maximum of *RE%* = 88 % within 20 and 50 mg L^{-1} (**SM-Section S6,** Figure S6**c**), which were real concentrations of polyphenols in honey samples, so, of outstanding importance for industrial application.

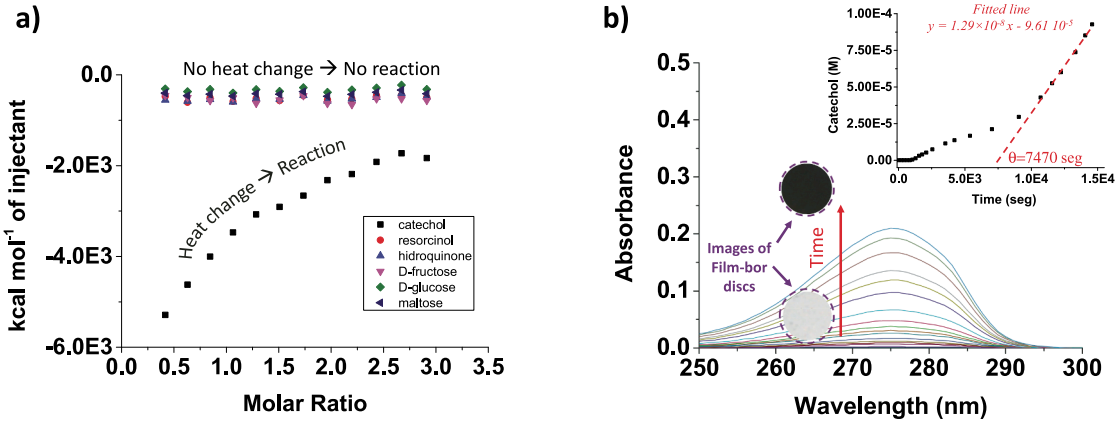

Fig. 5. Interaction of **Linear-bor** and **Film-bor** with catechol: a) isothermal titration calorimetry experiments. Sample cell contained an aqueous solution of **Linear-bor** at a starting concentration of phenylboronic acid groups of 0.61 mM. Different experiments with 9 mM solutions of catechol, resorcinol, hydroquinone, fructose, glucose, and maltose were carried out by adding 20 μL aliquots every 5 min. Temperature: 25 °C; and b) **Film-bor** membrane permeation study with catechol at 25 °C. Membrane thickness = 100 μm. Area = 2 cm^2. Content of beaker A = 200 mL of MQ water. Content of beaker B = 200 mL of a 5000 mg L^{-1} solution of catechol in Ultrapure water. The absorbance (at 275 nm) of beaker A was measured over time until a linear trend was achieved.

3.5. Proof of concept. GOX and CAT activity determination of 29 honey samples

This study was carried out on 29 honey samples from different botanical origins. The activity of both enzymes was measured in the original honey samples (without purification), as a demonstration of the need for samples to be purified. Additionally, the same 29 samples were also characterized after a dialysis process using Sigma-Aldrich dialysis membranes D6191, and after a purification process using **Film-bor**. The results obtained are shown in Table 1.

As shown in "As received" results, CAT activity could not be measured in many samples due to the interferents. This fact confirms the need to carry out a purification process in honey samples when determining GOX and CAT enzymatic activities.

However, it should be noted that either method is unlikely to remove 100 % of the polyphenols. At best, it will remove free polyphenols, since in honey, phenolic acids and flavonoids exist primarily in the form of soluble protein–polyphenol complexes or are incorporated into higher-order structures such as melanoidins. But, for practical purposes, these complexed polyphenols would not interfere in the measurement of catalase and GOX activity either.

There were no significant differences between the results of enzymatic activities obtained using the Sigma-Aldrich membrane and the **Film-bor** (commercial membrane vs **Film-bor**). Therefore, our proposed methodology using **Film-bor** is an excellent alternative to the actual procedure.

4. Conclusions

The actual interferents for reliably determining the honey's GOX and CAT activities are the catechol derivatives, so a film-shaped material extracts selectively this type of substances from honey samples has been designed, not compromising the rest of the original composition of the honey. The main goal of this study is a material that does not react with diols such as fructose, but reacts with 1,2-dihydroxybenzenes, such as catechol or quercetin, for which the role of polymeric chains has been determinant. New short-term research should be aimed at analyzing the extracted polyphenols by chromatographic techniques to determine the exact chemical structure of the extracted polyphenols, which would imply a previous hydrolysis step of the boronic esters formed in Film-bor. The results obtained with the proposed method are statistically equal to those obtained with the current purification method based on the use of a dialysis membrane, which postulates our material as a robust, simple, economical and eco-friendly alternative made with 100 % commercially available monomers, favouring its hypothetical industrial scaling and its use in the food industry and research.

5. Open data

Open Data is available at https://riubu.ubu.es/handle/10259/5684 (Dataset of the work *Straightforward purification method for the determination of the activity of glucose oxidase and catalase in honey by extracting polyphenols with a film-shaped polymer*).

CRediT authorship contribution statement

Lara González-Ceballos: Investigation, Conceptualization, Writing – original draft. **Jose Carlos Guirado-Moreno:** Investigation, Writing – original draft. **Gianluca Utzeri:** Investigation, Writing – original draft. **José Miguel García:** Writing – review & editing, Visualization. **Miguel A. Fernández-Muiño:** Conceptualization, Methodology. **Sandra M. Osés:** Conceptualization, Methodology, Investigation. **M. Teresa Sancho:** Writing – review & editing, Visualization. **Ana Arnaiz:** Investigation, Methodology, Writing – review & editing. **Artur J.M. Valente:** Conceptualization, Writing – review & editing, Visualization. **Saúl Vallejos:** Conceptualization, Methodology, Writing – original draft,

Table 1
Activity results for GOX and CAT expressed as "μg H_2O_2 g^{-1}_{honey}" and "g^{-1}_{honey} min^{-1}" respectively. The activity was measured in raw honeys, in dialyzed honeys using Sigma Aldrich dialysis membranes D6191 (Sigma), and in purified honeys using **Film-bor**. Data are means of two biological replicates ± standard deviation. Different letters indicate significant differences within the same row in GOX activity (upper case letters) or CAT activity (lower case letters). Two-way ANOVA followed by Uncorrected Fisher's LSD ($p < 0.05$) test was performed.

Sample number	GOX activity ($\mu g\ H_2O_2\ g^{-1}_{honey}$)			Catalase activity ($\times 10^{-3}$) ($g^{-1}_{honey}\ min^{-1}$)		
	As received	Sigma	Film–bor	As received	Sigma	Film–bor
1	nd	2.1 ± 1.2 A	7.7± 4.1 A	nd	3.2 ± 0.5 a	3.1 ± 2.1 a
2	10.2 ± 5.1 A	54.2 ± 50.4 A	38.6 ± 33.5 A	1.7 ± 0.2 a	66.23 ± 0.4c	62.2 ± 2.2b
3	998.7 ± 53.6 A	1121.8 ± 4.9B	1219.0 ± 75.2B	2.2 ± 0.3 a	15.1 ± 0.4b	13.5 ± 1.1b
4	124.2 ± 2.1 A	161.5 ± 5.3B	nd	nd	9.5 ± 0.6 a	7.8 ± 1.9 a
5	59.5 ± 3.4 A	90.2 ± 36.5 A	48.0 ± 3.0 A	nd	11.9 ± 0.2 a	11.4 ± 0.5 a
6	215.7 ± 0.9 A	264.5 ± 15.4 A	170.0 ± 26.8 A	36.6 ± 1.3 a	76.1 ± 0.2b	71.45 ± 1.3b
7	214.3 ± 3.4 A	330.2 ± 6.0 A	336.0 ± 6.0 A	3.5 ± 0.3 a	30.3 ± 1.2b	28.6 ± 0.0b
8	711.3 ± 36.7 A	903.2 ± 61.3B	914.4 ± 38.5B	nd	3.5 ± 0.2 a	3.9 ± 0.6 a
9	738.7 ± 4.8 A	923.9 ± 44.4B	1105.6 ± 228.3C	nd	36.3 ± 3.4b	12.6 ± 1.3 a
10	700.3 ± 8.2 A	886.0 ± 27.7B	898.3 ± 2.8B	11.2 ± 0.9 a	82.9 ± 1.2b	81.3 ± 2.5b
11	977.7 ± 51.9 A	1032.7 ± 97.8 A	1132.0 ± 1.9B	13.6 ± 3.3b	9.4 ± 3.4 a	8.9 ± 3.4 a
12	658.2 ± 3.0 A	652.0 ± 66.4 A	678.1 ± 39.4 A	nd	nd	2.1 ± 0.0
13	386.1 ± 1.7 A	513.4 ± 32.7 A	498.1 ± 46.9 A	nd	nd	2.3 ± 0.1
14	507.23 ± 76.6 A	874.9 ± 220.9B	927.3 ± 122.7B	nd	nd	nd
15	29.0 ± 29.6 A	615.9 ± 240.1B	655.1 ± 272.0B	nd	16.5 ± 0.5 a	16.8 ± 0.3 a
16	545.2 ± 111.4 A	1181.7 ± 18.9C	1034.0 ± 106.3B	2.3 ± 0.3 a	24.9 ± 1.1b	25.0 ± 0.7b
17	646.6 ± 64.2B	339.4 ± 110.8 A	461.2 ± 62.8 A	nd	6.0 ± 0.1 a	6.2 ± 0.2 a
18	504.5 ± 15.8 A	790.12 ± 41.3B	749.8 ± 54.7B	nd	nd	nd
19	441.8 ± 149.1 A	848.3 ± 38.9B	789.5 ± 35.0B	2.3 ± 0.6 a	17.1 ± 1.2c	12.9 ± 2.5b
20	478.4 ± 18.1B	600.8 ± 17.9B	236.6 ± 14.8 A	nd	3.9 ± 0.5 a	4.6 ± 1.1 a
21	115.5 ± 19.0 A	115.6 ± 27.7 A	102.9 ± 7.9 A	nd	5.8 ± 1.3 a	4.5 ± 0.5 a
22	364.4 ± 48.6 A		664.9 ± 13.8B	14.3 ± 3.8 a		27.23 ± 0.4b

(*continued on next page*)

Table 1 (*continued*)

Sample number	GOX activity ($\mu g\ H_2O_2\ g_{honey}^{-1}$)			Catalase activity ($\times 10^{-3}$) ($g_{honey}^{-1}\ min^{-1}$)		
	As received	Sigma	Film–bor	As received	Sigma	Film–bor
		657.6 ± 18.2B			25.12 ± 0.6b	
23	73.0 ± 10.2 A	196.8 ± 4.1 A	204.4 ± 11.6 A	nd	nd	nd
24	11.0 ± 5.3 A	6.4 ± 0.3 A	6.0 ± 0.9 A	nd	nd	nd
25	488.5 ± 23.7 A	459.0 ± 9.1 A	697.4 ± 3.9B	nd	10.8 ± 0.6 a	10.3 ± 0.1 a
26	366.2 ± 9.1 A	530.4 ± 1.5B	523.7 ± 9.1B	10.2 ± 1.8 a	14.4 ± 0.5b	13.1 ± 0.6 ab
27	484.7 ± 50.5 A	590.6 ± 36.3 A	612.8 ± 10.2 A	nd	nd	nd
28	Nd	1494.3 ± 9.4 A	1490.4 ± 6.9 A	nd	4.34 ± 0.1 a	4.2 ± 0.2 a
29	186.8 ± 29.5 A	421.8 ± 53.7B	387.4 ± 55.6B	nd	2.9 ± 0.2 a	3.0 ± 0.2 a

Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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