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Green extraction of phenolic compounds from olive pomace: optimization of autohydrolysis conditions and evaluation of potential applications

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SUMMARY: This study evaluated the use of autohydrolysis as a green method to extract biophenols with antioxidant and antibiofilm activities from Uruguayan olive pomace (OP), considering the formation of degradation compounds such as 5-hydroxymethylfurfural (HMF). Response surface methodology was used to evaluate the effect of temperature, extraction time and liquid-solid ratio on extraction yield (EY), total phenolic content (TPC), antioxidant activity (FRAP and DPPH assays), hydroxytyrosol (HTY) content and HMF content. The optimal extraction conditions (168 °C, 24 min, and liquid-solid ratio of 20 mL/g) were determined to maximize EY (25.65 g/100 g), TPC (28.60 mgGAE/g), antioxidant activity (FRAP: 328.50 µmolFSE/g; DPPH: 122.33 µmolTRE/g) and HTY content (2.79 mg/g) while minimizing HMF content (0.92 mg/g). Under these conditions, the obtained extract showed greater protection for sunflower oil than the synthetic antioxidants tested (BHA and BHT) and demonstrated the ability to inhibit the biofilm formation of *Candida albicans* and *Staphylococcus aureus*.

KEY-WORDS: *5-hydroxymethylfurfural; Antibiofilm; Antioxidants; Autohydrolysis; Hydroxytyrosol; Olive pomace.*

RESUMEN: ***Extracción ecológica de compuestos fenólicos de orujo de aceituna: optimización de las condiciones de autohidrólisis y evaluación de aplicaciones potenciales.*** Este estudio evaluó el uso de la autohidrólisis como método ecológico para la extracción de biofenoles con actividad antioxidante y antibiofilm del orujo de aceituna (OP) uruguayo, considerando la formación de compuestos de degradación como el 5-hidroximetilfurfural (HMF). Se empleó la metodología de superficie de respuesta para evaluar el efecto de la temperatura, el tiempo de extracción y la relación líquido-sólido sobre el rendimiento de extracción (EY), el contenido fenólico total (TPC), la actividad antioxidante (ensayos FRAP y DPPH), el contenido de hidroxitirosol (HTY) y el contenido de HMF. Se determinaron las condiciones óptimas de extracción (168 °C, 24 min y una relación líquido-sólido de 20 mL/g) para maximizar el EY (25,65 g/100 g), el TPC (28,60 mgGAE/g), la actividad antioxidante (FRAP: 328,50 µmolFSE/g; DPPH: 122,33 µmolTRE/g) y el contenido de HTY (2,79 mg/g), a la vez que se minimizaba el contenido de HMF (0,92 mg/g). En estas condiciones, el extracto obtenido mostró una mayor protección para el aceite de girasol que los antioxidantes sintéticos estudiados (BHA y BHT) y demostró su capacidad para inhibir la formación de biopelículas de *Candida albicans* y *Staphylococcus aureus*.

PALABRAS CLAVE: *5-hidroximetilfurfural; Antibiopelícula; Antioxidantes; Autohidrólisis; Hidroxitirosol; Orujo de oliva.*

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1. INTRODUCTION

The olive oil extraction process generates significant waste with high phytotoxicity, posing environmental risks to land and water ecosystems (Quero *et al.*, 2022). Under the two-phase extraction system, the primary by-product is olive pomace or alperujo (OP), a semi-solid slurry comprising of olive skin, pulp, pit, and water, with a moisture content of 60-70 % (Alburquerque *et al.*, 2004). Notably, 98-99 % of the fruits' biophenols remain in OP post-extraction, making it a valuable source of natural antioxidants (Ghanbari *et al.*, 2012). Hydroxytyrosol (HTY) is the predominant polyphenol in OP, accompanied by tyrosol, 3,4-dihydroxyphenylglycol, various phenolic acids (*p*-coumaric, vanillic, and caffeic acids), flavonoids (rutin), and secoiridoid complexes like verbascoside and oleuropein derivatives (Rubio-Senent *et al.*, 2012). These compounds exhibit antioxidant, anti-inflammatory, antimicrobial, antihypertensive, and cardioprotective properties (Ghanbari *et al.*, 2012). Utilizing natural antioxidants from OP presents promising applications in the food and pharmaceutical industries (Araújo *et al.*, 2015).

Extracting bioactive compounds is a critical step to obtaining natural antioxidants. Conventional solid-liquid extraction using organic solvents like methanol, ethanol, and ethyl acetate is widely employed (Cabrera *et al.*, 2024). However, drawbacks such as toxicity, high solvent requirements, volatility, and flammability necessitate greener alternatives. One such alternative is autohydrolysis, which eliminates organic solvents by using water alone (Ballesteros *et al.*, 2017). When lignocellulosic material is exposed to water or steam at 150-230 °C, autohydrolysis occurs due to water self-ionization and the release of acidic compounds (Garrote *et al.*, 1999). This process depolymerizes hemicelluloses and partially deconstructs lignin, facilitating phenolic compound solubilization (Rubio-Senent *et al.*, 2012).

Research has demonstrated the effectiveness of autohydrolysis in extracting biophenols with antioxidant activity from OP (Lama-Muñoz *et al.*, 2019; Rubio-Senent *et al.*, 2012). However, optimal extraction conditions remain unexamined, particularly regarding the influence of temperature, extraction time, and solid-liquid ratio, as well as their interactions. Additionally, the formation of degradation compounds such as 5-hydroxymethylfurfural (HMF) remains understudied. Optimizing extraction parameters is essential for maximizing biophenol yields while minimizing HMF formation, especially given its adverse health effects (Pastoriza de la Cueva *et al.*, 2017). HMF serves as a marker for Maillard reaction-derived toxicants in heat-treated foods, correlating with acrylamide and other furan formation, while being easier and more cost-effective to measure (Mariotti-Celis *et al.*, 2018).

Concerns over synthetic additives in the food industry, particularly due to potential health risks from prolonged exposure, have driven demand for natural alternatives. Edible oils commonly contain synthetic antioxidants such as butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) (Dauber *et al.*, 2022b). While effective and cost-efficient, their toxicity has led to restrictions. Consequently, the use of natural additives from olive oil by-products offers an opportunity for food and beverage companies to meet consumer demand while addressing these challenges (Nunes *et al.*, 2021).

Evaluating the antibiofilm activity of biophenols extracted from OP is crucial due to their potential for combating biofilm-associated infections. Biofilms contribute to recurrent and chronic infections, including antibiotic-resistant strains, with 99 % of bacteria existing as biofilms. These microorganisms colonize medical devices such as heart valves and catheters, significantly contributing to healthcare-associated infections, which increase morbidity, mortality, costs, and hospital stays. Biofilms can be up to 1000 times more resistant than planktonic bacteria (Raffaelli *et al.*, 2022). Studies suggest that certain biophenols inhibit biofilm formation or disrupt existing biofilms, offering a natural antimicrobial approach (Slobodníková *et al.*, 2016). This highlights new opportunities for sustainable utilization of OP-derived phenolics in antimicrobial formulations.

In this context, this study aimed to explore the influence of extraction conditions (temperature, extraction time, and liquid-solid ratio) on biophenol extraction from OP using autohydrolysis. The study also sought to optimize these parameters to maximize antioxidant extraction while minimizing HMF formation. Furthermore, the potential applications of extracts obtained under optimal conditions were evaluated, focusing on their ability to stabilize sunflower oil and their antibiofilm properties.

2. MATERIALS AND METHODS

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ 99 %), (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxilic acid (Trolox > 98 %), BHA (99 %), BHT (99 %), HTY (analytical standard) and HMF (analytical standard) were purchased from Sigma Aldrich (St. Louis, USA). Folin-Ciocalteu reagent, sodium carbonate anhydrous, acetonitrile (HPLC grade) and gallic acid (99 %) were purchased from Merck (Darmstadt, Germany). Ferric chloride hexahydrate was purchased from Fluka Analytical (St. Louis, USA). Sodium acetate anhydrous, trifluoroacetic acid and glacial acetic acid were purchased from Carlo Erba Reagents (Val de Reuil, France). Ferrous sulfate heptahydrate was purchased from Cicarelli Reagents (Santa Fe, Argentina). Nutrient broth and potato dextrose broth were purchased from Oxoid (Basingstoke, UK).

2.2. Raw materials

OP, consisting of a 50 % blend of Arbequina and Picual varieties (harvested at maturity index between 3.5 and 4), was sourced from a local olive oil mill (Canelones, Uruguay). It was obtained using a two-phase extraction system. Fresh samples were collected, portioned into plastic bags, and immediately stored at -18 °C for later use.

2.3. Extraction and optimization methodology

2.3.1. Autohydrolysis process

Autohydrolysis experiments were performed in a 2-L agitated cylindrical stainless-steel reactor (AmAr Equipment, India) equipped with an electric resistance heating system with temperature control and an internal coil cooling system fed with tap water.

For each experiment, distilled water and OP were added to the reactor, with agitation at 220 rpm. Temperature, extraction time, and liquid-solid ratio were controlled as described further in this section, considering the water content of OP. Heating from ambient to the target temperature took 30–40 min, with operating pressure matching the system's equilibrium pressure. Extraction time began upon reaching the target temperature. After extraction, the reactor was cooled for 10 min to ~30 °C, then centrifuged (2500 g, 10 min). The liquid fraction was filtered (0.45 μm) and stored at -18 °C. Extraction yield (EY) was determined by drying the liquid extract in an oven at 105 °C until constant weight and expressed as g of solid extract per 100 g of OP (dry basis).

For the oxidative stability and antibiofilm activity analyses described in Section 2.4, a fraction of the liquid extract obtained as previously described was dried in a laboratory spray dryer (model HT-RY1500, Zhengzhou Hento Machinery, Henan, China) without encapsulants, under the following conditions: inlet air temperature to the chamber: 150 °C; liquid extract feed flow rate: 10 mL/min; air feed fan: 70 % of the nominal maximum flow rate. The powdered extract was stored in airtight amber glass vials until further analysis.

2.3.2. Experimental design and optimization methodology

For autohydrolysis process modeling, Response Surface Methodology (RSM) was applied using a three-factor, three-level Box-Behnken design. The temperature of the independent variables (Y_1: 145, 157.5, and 170 °C), extraction time (Y_2: 15, 37.5, and 60 min), and liquid-solid ratio (Y_3: 5, 12.5, and 20 mL/g, dry basis), were evaluated for their effects on extraction yield (EY), total phenolic content (TPC), antioxidant activity (Ferric reducing antioxidant power, FRAP assay, and free radical scavenging capacity assay using 2,2-diphenyl-1-picrylhydrazyl reagent, DPPH assay), HTY content, and HMF content. Variable levels were

determined based on preliminary experiments (data not shown). Fifteen experiments, including three center-point replicates, were performed in a randomized order, with responses modeled according to:

$$z = \alpha_0 + \sum_{i=1}^{3} \alpha_i Y_i + \sum_{i=1}^{3} \alpha_{ii} Y_i^2 + \sum_{i=1}^{2} \sum_{j=2}^{3} \alpha_{ij} Y_i Y_j$$

where α_0 is the constant term, α_i is the linear term, α_{ii} is the quadratic term, and α_{ij} is the interaction term, all corresponding to the dependent variable z. Y_i and Y_j are the levels of the independent variables coded according to:

$$Y_i = \frac{2\,(y_i - y_{Mi})}{\Delta y_i}$$

where y_i and Y_i denote the actual and encoded values of the independent variable i, respectively; y_{Mi} represents the value of the independent variable i at the central point, and Δy_i signifies the range of variation for the independent variable i. All extractions and subsequent separations were carried out using the previously described experimental setup.

To optimize the key parameters influencing the extraction process, the desirability function methodology proposed by Derringer and Suich (1980) was applied. The optimization aimed to simultaneously maximize the responses EY, TPC, antioxidant activity (FRAP and DPPH), and HTY content while minimizing HMF content, considering the independent variables temperature, extraction time, and liquid-solid ratio.

2.4. Analytical methods

2.4.1. Raw OP characterization

The characterization of the raw OP was carried out through the following determinations: water content was analyzed gravimetrically at 105 °C according to Cabrera *et al.* (2024); total organic matter and ashes were determined gravimetrically at 550 °C according to Rodriguez *et al.* (2023); total fat content was analyzed by extraction with n-hexane/isopropanol (3:2 v/v) according to Hara and Radin (1978); initial total phenolic content was determined by exhaustive multistage extraction with methanol 80 % (v/v) according to Xavier *et al.* (2022).

2.4.2. Total phenolic content

TPC was measured using the Folin-Ciocalteu method (Singleton and Rossi, 1965), following the procedure outlined by Xavier *et al.* (2014). Briefly, a 0.5 mL aliquot of the extract solution was mixed with 2.5 mL of 1:10 diluted Folin–Ciocalteu reagent and 2 mL of 75 g/L sodium carbonate solution. The mixture was incubated at 50 °C for 5 minutes, cooled, and the absorbance was measured at 760 nm using a Shimadzu UVmini-1240 spectrophotometer. A calibration curve made of standard solutions of gallic acid (10–50 mg/L) was used. The results were reported as milligrams of gallic acid equivalents per gram of OP on a dry basis (mg GAE/g).

2.4.3. Antioxidant activity

The FRAP assay was performed according to the method outlined by Piwowarska and González-Álvarez (2012) with minor modifications. Briefly, 3.0 mL of freshly prepared FRAP reagent—comprising of 2.5 mL of 10 mmol/L TPTZ in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3$, and 25 mL of 300 mmol/L acetate buffer (pH 3.6)—was mixed with 0.1 mL of the diluted extract and incubated for 10 min at 25 °C. Absorbance was then measured at 593 nm using a Shimadzu UVmini-1240 spectrophotometer. A calibration curve made of standard solutions of $FeSO_4 \cdot 7\ H_2O$ (100–1000 µmol/L) was used. The results were reported as micromoles of ferrous sulfate equivalents per gram of OP on a dry basis (µmol FSE/g).

The DPPH assay was conducted following the method described by Cabrera *et al.* (2024). In short, 0.3 mL of the previously diluted extract was mixed with 2.7 mL of a freshly prepared DPPH solution (6.0 x 10^{-5} mol/L in 80 % (v/v) methanol). After incubating in the dark at room temperature for 30 minutes, the absorbance was recorded at 517 nm (UVmini-1240 spectrophotometer). A calibration curve made of standard solutions of Trolox (40−750 µmol/L) in 80 % (v/v) methanol was used. The results were reported as micromoles of Trolox equivalents per gram of OP on a dry basis (µmol TRE/g).

2.4.4. High performance liquid chromatography (HPLC)

The HTY and HMF content analysis was performed by HPLC with the equipment and procedure outlined by Cabrera *et al.* (2024). The mobile phases consisted of 0.01 % trifluoroacetic acid in Milli-Q water (solvent A) and acetonitrile (solvent B), with the following gradient: 0–30 min, 95–75 % A; 30–45 min, 75–50 % A; 45–47 min, 50–0 % A; 47–50 min, 0–75 % A; 50–52 min, 75–95 % A, followed by isocratic conditions until 55 min. The injection volume was 20 µL, the flow rate 1.0 mL/min, and the column temperature 25 °C. The quantification was performed by integrating peaks at 280 nm, utilizing external standard calibration curves (HTY: 1-120 mg/L; HMF: 1-100 mg/L). The results were reported in milligrams per gram of OP on a dry basis (mg/g).

2.4.5. Oxidative stability evaluation

The efficiency of the extract obtained under optimal extraction conditions for protecting sunflower oil (SFO) against an accelerated oxidation process was studied using an 873 Biodiesel Rancimat (Metrohm, Switzerland) according to the procedure reported by Vieitez *et al.* (2018). Briefly, the selected OP extract was dispersed in absolute ethanol, vortexed, and sonicated in a water bath for 30 min, then incorporated into the commercial SFO at the following concentrations: 500, 1000, 2500 and 5000 ppm (mass of extract/mass of SFO). In all cases complete solubilization was achieved. The induction period (IP) of the oxidation process was determined at 100 °C, under airflow (0.02 m^3/h). The results were compared with SFO activated with 200 ppm of BHT and 100 ppm of BHA. This procedure was performed in triplicate according to the AOCS technique Cd-12b-92 (AOCS, 1997).

2.4.6. Antibiofilm activity

The antibiofilm assays were carried out using standard strains of microorganisms: *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 15442, *Candida albicans* ATCC 101231, and *Escherichia coli* ATCC 25922. These strains were cryo-conserved at -80 °C in the Microbiology Laboratory at Facultad de Química, UdelaR, Uruguay, and previously reported as biofilm formers (Estevez *et al.*, 2020; Raffaelli *et al.*, 2022). Biofilm inhibitory ability was determined according to Raffaelli *et al.* (2022). For bacterial testing, serial dilutions of the extract in nutrient broth (NB) were prepared in a 96-well Nunc™ MicroWell™ plate, followed by the addition of a standardized bacterial suspension (3 × 10^8 cells/mL). Control wells included sterile broth (blank) and sterile broth with bacterial suspension (non-treated biofilm, NTB). The plate was then incubated at 37 °C for 24 h. After incubation, the supernatant was removed, and the remaining biofilm was washed with distilled water and dried at 50 °C for 40 minutes. The biofilm mass was then stained with a 1 % crystal violet solution for 3 minutes, rinsed with water, resuspended in an ethanol-acetone solution (70:30), and its absorbance was measured at 590 nm. Each concentration, as well as control wells, were done in quadruplicates, at least. For *Candida albicans*, a standardized yeast solution (3x10^6 cells/mL) was placed in each well (except sterile control wells) and incubated for 30 min at 37 °C. The supernatant was removed and the wells were washed with PBS 1x. Then, serial dilutions of the extract in potato dextrose broth (PDB) were placed. Control wells containing sterile broth (blank) and sterile broth + yeast suspension (NTB) were included. Then, the procedure was continued as indicated for the bacterial assay. The absorbances were compared and the inhibition percentage calculated.

2.5. Statistical analysis

Tukey test, analysis of variance (ANOVA), Pearson correlation coefficient, and response surface analysis were conducted using RStudio 2024.12.0, all with a 95 % confidence level. Contour plots were generated using PGF/TikZ 3.1.10. Desirability function and optimization were performed using GNU Octave version 7.1.0.

3. RESULTS AND DISCUSSION

3.1. Characterization of raw OP

Raw OP was characterized by a water content of 71.7 ± 0.6 % on a wet basis; total organic matter of 27.1 ± 0.8 % on a wet basis; ash content of 1.3 ± 0.0 % on a wet basis; total fat content of 5.3 ± 0.1 % on a wet basis; and initial total phenolic content of 14.48 ± 0.55 milligrams of gallic acid equivalents per gram of OP on a dry basis. In general, all values are similar to those reported by other researchers (Alburquerque *et al.*, 2004; Gimenez *et al.*, 2020; Rodriguez *et al.*, 2023). However, it is worth highlighting the high initial content of total phenols in the OP used in the present work compared to that reported by Rodriguez *et al.* (2023), whose OP came from a geographically close area to that of the present study. In summary, OP is a by-product with high moisture and organic matter content, and an interesting level of total phenolic compounds, making it suitable for potential utilization.

Regarding the residual oil content in the fresh olive pomace, it should be noted that after the extractions, as described in Section 2.3, the extract was filtered using 0.45 μm filters. In this way, all the oil was retained in the filter, leaving the extracts free of oil.

3.2. Modelling of the autohydrolysis process

RSM was used to assess the effects of temperature, extraction time, and liquid-solid ratio on EY, TPC, FRAP and DPPH, as well as HTY and HMF contents. Table 1 summarizes the experimental conditions and results. The highest EY, TPC, and antioxidant activity were observed at maximum temperature and liquid-solid ratio with an intermediate extraction time, whereas the lowest values occurred under the mildest conditions. HMF content peaked at the highest temperature and extraction time and was lowest under the mildest conditions, both at an intermediate liquid-solid ratio.

TABLE 1. Experimental conditions and results for biophenol extraction from OP using a Box-Behnken design

Exp	Variables[a]			Responses[b]					
	Y_1	Y_2	Y_3	EY	TPC	FRAP	DPPH	HTY	HMF
1	145 (-1)	15 (-1)	12.5 (0)	24.77	18.60	215.06	97.95	2.19	0.04
2	170 (1)	15 (-1)	12.5 (0)	26.16	24.37	283.86	106.52	2.49	0.61
3	145 (-1)	60 (1)	12.5 (0)	23.13	20.57	255.45	105.58	2.47	0.28
4	170 (1)	60 (1)	12.5 (0)	28.15	27.85	327.71	113.40	3.08	2.51
5	145 (-1)	37.5 (0)	5 (-1)	22.46	18.57	208.73	83.50	1.95	0.16
6	170 (1)	37.5 (0)	5 (-1)	25.28	23.20	257.39	92.85	2.12	1.71
7	145 (-1)	37.5 (0)	20 (1)	25.14	21.54	258.12	111.04	2.18	0.12
8	170 (1)	37.5 (0)	20 (1)	28.89	29.32	350.52	127.18	2.92	1.65
9	157.5 (0)	15 (-1)	5 (-1)	21.77	18.84	210.92	88.55	1.96	0.19
10	157.5 (0)	60 (1)	5 (-1)	23.30	21.98	252.61	91.73	3.38	0.98
11	157.5 (0)	15 (-1)	20 (1)	25.47	23.40	285.96	114.05	2.66	0.17
12	157.5 (0)	60 (1)	20 (1)	27.02	26.81	338.14	127.97	2.33	1.08

13	157.5 (0)	37.5 (0)	12.5 (0)	23.67	23.93	282.62	106.54	2.90	0.53
14	157.5 (0)	37.5 (0)	12.5 (0)	25.35	24.66	295.11	106.55	3.00	0.55
15	157.5 (0)	37.5 (0)	12.5 (0)	24.87	25.11	285.57	111.60	3.09	0.59

[a] Y_1: temperature (°C); Y_2: extraction time (min); Y_3: liquid-solid ratio (mL/g). Real and (coded) values.
[b] EY: extraction yield (g/100 g); TPC: total phenolic content (mg GAE/g); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (μmol FSE/g); DPPH: antioxidant activity by the free radical scavenging capacity assay (μmol TRE/g); HTY: hydroxytyrosol content (mg/g); HMF: 5-hydroxymethylfurfural content (mg/g).

The ANOVA results (Table 2) confirm that all models were highly significant ($p < 0.0001$, except for HTY at p=0.0017), with non-significant lack-of-fit values ($p > 0.05$), validating their predictive accuracy. Table 3 presents the regression coefficients and determination coefficients for all models after removing non-significant terms ($p > 0.05$). All six models showed high values for the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}). The predicted determination coefficients (R^2_{pred}) closely aligned with R^2_{adj}, demonstrating strong agreement between experimental and predicted values. For the HMF model, a natural logarithm transformation (Y replaced by ln (Y) in Equation 1) was applied to enhance model fit. Overall, the models effectively capture the influence of process variables on the response variables.

TABLE 2. Analysis of variance for the reduced response surface models

Response[a]	Source	Sum of square	Degrees of freedom	Mean square	F-value	p-value
EY	Model	53.09	5	10.62	32.43	<0.0001
	Residual	2.95	9	0.33		
	Lack of fit	1.44	7	0.21	0.27	0.9175
	Pure error	1.50	2	0.75		
TPC	Model	151.00	7	21.57	99.92	<0.0001
	Residual	1.51	7	0.22		
	Lack of fit	0.81	5	0.16	0.46	0.7929
	Pure error	0.71	2	0.35		
FRAP	Model	26622.74	7	3803.25	158.45	<0.0001
	Residual	168.02	7	24.00		
	Lack of fit	82.84	5	16.57	0.39	0.8293
	Pure error	85.18	2	42.59		
DPPH	Model	2253.94	3	751.31	78.64	<0.0001
	Residual	105.09	11	9.55		
	Lack of fit	88.05	9	9.78	1.15	0.5491
	Pure error	17.05	2	8.52		
HTY	Model	2.50	5	0.50	10.17	0.0017
	Residual	0.44	9	0.05		
	Lack of fit	0.42	7	0.06	6.26	0.1447
	Pure error	0.02	2	0.01		
HMF[b]	Model	2.28	5	0.46	391.35	<0.0001
	Residual	0.01	9	0.001		
	Lack of fit	0.01	7	0.001	3.66	0.2316
	Pure error	0.001	2	0.0004		

[a]EY: extraction yield (g/100 g); TPC: total phenolic content (mg GAE/g); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (μmol FSE/g); DPPH: antioxidant activity by the free radical scavenging capacity assay (μmol TRE/g); HTY: hydroxytyrosol content (mg/g); HMF: 5-hydroxymethylfurfural content (mg/g). [b]After applying natural logarithm transformation.

3.3. Influence of extraction conditions on EY, TPC and antioxidant activity

As shown in Table 3, EY was affected by extraction temperature (with significant linear and quadratic terms), extraction time (with significant linear term), and liquid-solid ratio (with significant linear term), with statistically significant interaction between temperature and extraction time. TPC was influenced by extraction temperature, extraction time, and liquid-solid ratio, all with significant linear and quadratic terms, and statistically significant interaction between temperature and liquid-solid ratio. Antioxidant activity measured using the FRAP method followed the same pattern as TPC, with significant linear and quadratic terms for extraction temperature, extraction time, and liquid-solid ratio, and statistically significant interaction between temperature and liquid-solid ratio. For antioxidant activity measured with DPPH, only the linear contributions of temperature, extraction time, and liquid-solid ratio were statistically significant, while the interactions of the independent variables had no significant effect.

TABLE 3. Regression and determination coefficients for the reduced response surface models (in coded variables)

	EY	TPC	FRAP	DPPH	HTY	HMF[a]
α_0	24.492***	24.566***	287.765***	105.667***	2.950***	-0.568***
α_0	1.623***	3.182***	35.266***	5.234***	0.228*	1.245***
α_2	0.429*	1.500***	22.265***	3.952**	0.246*	0.872***
α_3	1.713***	2.310***	37.885***	15.451***	—	—
α_{12}	0.907*	—	—	—	—	-0.159**
α_{13}	—	0.789*	10.935**	—	—	—
α_{23}	—	—	—	—	-0.439**	—
α_{11}	1.007**	-0.657*	-10.230**	—	-0.358*	-0.166**
α_{22}	—	-1.059**	-7.016*	—	—	-0.294***
α_{33}	—	-0.749*	-8.843*	—	-0.332*	—
R^2	0.947	0.990	0.994	0.956	0.850	0.997
R^2_{adj}	0.918	0.980	0.988	0.943	0.766	0.995
R^2_{pred}	0.858	0.966	0.971	0.920	0.629	0.992

EY: extraction yield (g/100 g); TPC: total phenolic content (mg GAE/g); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (µmol FSE/g); DPPH: antioxidant activity by the free radical scavenging capacity assay (µmol TRE/g); HTY: hydroxytyrosol content (mg/g); HMF: 5-hydroxymethylfurfural content (mg/g). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. [a]The model is for the transformed response: natural logarithm of HMF (ln (HMF)).

To illustrate the impact of extraction conditions on the response variables, contour plots are presented in Figure 1. The liquid-solid ratio was fixed at 20 mL/g, as this yielded the highest values for all response variables. This aligns with mass transfer principles, where a greater concentration gradient between the solid and liquid phases enhances the extraction of biophenols (Pinelo *et al.*, 2005). However, a high liquid-solid ratio in extraction processes can present challenges when scaling up the process, particularly from an economic standpoint (Elmas *et al.*, 2025). Nevertheless, when analyzing the extraction conditions in the present study, it should be noted that a large portion of the water used in the extraction comes from the fresh OP itself, given its high initial moisture content. Considering the initial water content of OP, the higher liquid-solid ratio used corresponds to 5 mL of added water per gram of fresh OP.

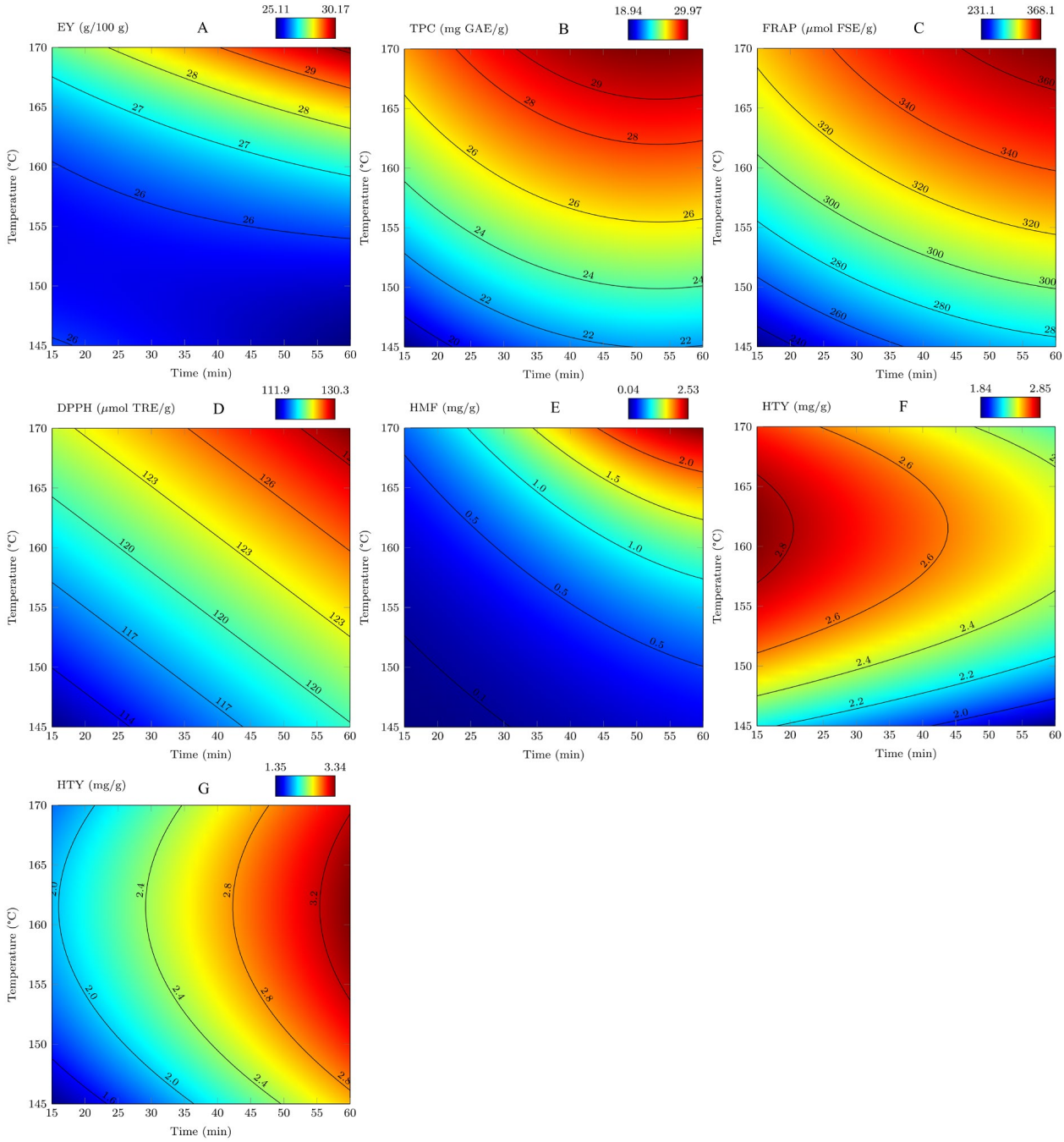

FIGURE 1. Contour plots representing EY (A), TPC (B), antioxidant activity: FRAP (C) and DPPH (D), and HMF content (E) of extracts from OP as a function of extraction temperature and time at liquid-solid ratio of 20 mL/g. Contour plots representing HTY content of extracts from OP as a function of extraction temperature and time at liquid-solid ratio of 20 mL/g (F) and liquid-solid ratio of 5 mL/g (G).

The contour plots show that increasing extraction temperature improves EY, TPC, and antioxidant activity. Similarly, extraction time has a positive effect, though its impact diminishes at longer durations, particularly for TPC and FRAP antioxidant activity. These findings align with previous studies on autohydrolysis applied to olive pomace (Lama-Muñoz *et al.*, 2019; Rubio-Senent *et al.*, 2012) and other agro-industrial by-products (Ballesteros *et al.*, 2017). Higher temperatures reduce water's polarity by disrupting hydrogen bonds, making it behave more like organic solvents (e.g., methanol or ethanol), thus enhancing the solubility of biophenols (Ballesteros *et al.*, 2017). Additionally, the combined effect of temperature and extraction time facilitates

hemicellulose depolymerization, releasing bound phenolic compounds with antioxidant activity (Rubio-Senent *et al.*, 2012). However, at extended extraction times, the increasing trend in TPC levels off, as seen in Figure 1A. This may be due to an initially high solubilization rate driven by a strong mass transfer gradient and the abundance of easily extractable biophenols. Over time, the available phenolics decrease, slowing diffusion into the liquid phase (Zakaria *et al.*, 2022). Additionally, the degradation of certain phenolics at high temperatures (Section 3.4) may further contribute to this trend.

The results further indicate a strong similarity between the contribution of extraction variables on TPC and antioxidant activity measured by FRAP, as evidenced by a high positive linear correlation (Pearson correlation coefficient of 0.976). Positive correlations also exist between TPC and antioxidant activity measured by DPPH, as well as between both antioxidant assays (Pearson correlation coefficients of 0.809 and 0.894, respectively). The antioxidant activity of biophenols is influenced not only by the properties of individual molecules but also by their interactions (Araújo *et al.*, 2015). Consequently, the solvent characteristics and extraction conditions can lead to variations in antioxidant activity and TPC assessments. The findings of this study suggest that the biophenols in OP extracts significantly contribute to the extracts' antioxidant activity, as assessed by both FRAP and DPPH assays. However, this relationship is not always observed, as demonstrated in a previous study by our research group (Cabrera *et al.*, 2024).

3.4. Influence of extraction conditions on HMF and HTY contents

As presented in Table 3, the HMF content was affected by extraction temperature and extraction time, both of which had significant linear and quadratic effects. The liquid-solid ratio had a statistically insignificant impact, while the interaction between temperature and extraction time showed a statistically significant influence. In the case of HTY content, this response was influenced by extraction temperature (with significant linear and quadratic terms), extraction time (with significant linear term) and liquid-solid ratio (with significant quadratic term), along with a statistically significant impact from the interaction between extraction time and the liquid-solid ratio.

Based on the contour plot shown in Figure 1E, HMF content increases with rising extraction temperature and time. The liquid-solid ratio was maintained at the maximum experimental value (20 mL/g) as in the case of the previously discussed response variables, since the influence of the liquid-solid ratio was statistically insignificant. A practically negligible HMF content was obtained under the least severe experimental conditions (145 °C and 15 min), while the highest experimental value was recorded for the conditions of maximum temperature and extraction time (170 °C and 60 min). This was an expected outcome, as the formation of HMF has been reported when extraction processes are applied at relatively high temperatures, both in by-products of the olive oil industry (Herrero *et al.*, 2012; Rubio-Senent *et al.*, 2012) as well as in other agro-industrial by-products (Mariotti-Celis *et al.*, 2018).

As shown in the contour plots (Figures 1F and 1G), HTY content increases with temperature up to approximately 160 °C, then declines. Extraction time has an inverse effect compared to other response variables, with HTY content decreasing as time increases, though this trend is only evident at a liquid-solid ratio of 20 mL/g. At lower ratios (Figure 1G), HTY content instead rises with extended extraction time, while the temperature effect remains consistent. The highest HTY content was observed at 157.5 °C (intermediate temperature), 60 min (maximum time), and 5 mL/g (minimum liquid-solid ratio), while the lowest occurred at 145 °C, 37.5 min, and 5 mL/g, similar to values at 157.5 °C and 15 min. These results suggest two competing processes: HTY diffusion into the solvent, either freely or as part of hemicellulose, and partial HTY degradation under high temperatures. The extent of these effects depends on temperature, time, and liquid-solid ratio, with higher liquid-solid ratios favoring degradation.

Previous studies have reported increases in HTY content when olive pomace was subjected to autohydrolysis extraction using steam at similar temperatures and times (Gimenez *et al.*, 2020; Lama-Muñoz *et al.*, 2019; Rubio-Senent *et al.*, 2012), as well as at lower temperatures (Rodriguez *et al.*, 2023). In these studies, liquid-solid ratios ranged from 2 to 8 mL/g, aligning with the trends observed in the present work. However, to the best of our knowledge, the influence of temperature and extraction time on HTY content at high liquid-solid ratios in the autohydrolysis processes has not been previously reported.

3.5. Optimization of extraction conditions and model validation

Figure 2 presents the contour plot illustrating the overall desirability as a function of extraction time and temperature, at a liquid-solid ratio of 20 mL/g. As mentioned in Section 3.4, higher HTY content is achieved at liquid-solid ratios below 20 mL/g. However, since the optimization goal is to simultaneously maximize EY, TPC, antioxidant activity (FRAP and DPPH), and HTY content, while minimizing HMF content, four of these variables (EY, TPC, FRAP, and DPPH) reach their maximum values at the highest liquid-solid ratio. Therefore, the relative weight of these variables in the overall desirability function is greater when using the maximum experimental liquid-solid ratio compared to a lower ratio that maximizes HTY content.

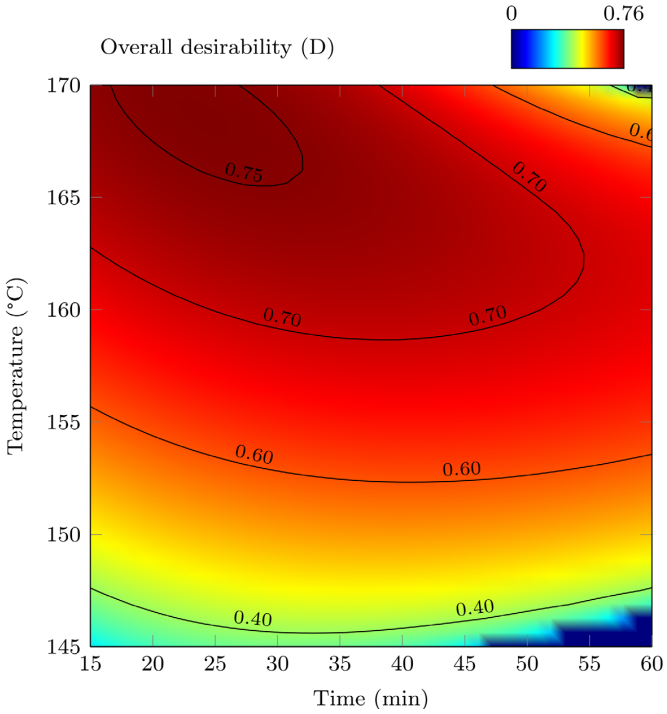

FIGURE 2. Contour plot depicting the overall desirability as a function of extraction temperature and time at a liquid-solid ratio of 20 mL/g.

As shown in Figure 2, there is a zone where the highest values of the overall desirability function are achieved (values around 0.75-0.76). This corresponds to extraction temperatures between 167-169 °C and extraction times between 22-27 min. It is important to highlight that the minimization of the HMF content variable is what causes the extraction temperature and time values to be lower than the maximum experimental values used in this study, with the reduction in extraction time having more weight than the reduction in temperature. This is consistent with the analysis in Sections 3.3 and 3.4.

To validate the RSM models, three extractions were performed under optimal conditions: 168 °C, 24 min, and a 20 mL/g liquid-solid ratio. Table 4 compares the experimental results with the model-predicted values. All experimental values fell within the 95 % confidence intervals of the predictions, confirming the models' accuracy in describing the extraction process.

TABLE 4. Experimental and predicted values by the models at the optimal extraction conditions

	EY	TPC	FRAP	DPPH	HTY	HMF
Experimental	25.65 ± 0.17	28.60 ± 0.86	328.50 ± 1.65	122.33 ± 3.24	2.79 ± 0.14	0.92 ± 0.01
Predicted	27.68 ± 0.84	27.84 ± 0.86	333.75 ± 9.04	123.36 ± 3.92	2.66 ± 0.31	0.87 ± 0.09

EY: extraction yield (g/100 g); TPC: total phenolic content (mg GAE/g); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (μmol FSE/g); DPPH: antioxidant activity by the free radical scavenging capacity assay (μmol TRE/g); HTY: hydroxytyrosol content (mg/g); HMF: 5-hydroxymethylfurfural content (mg/g). Experimental values are expressed as the mean ± standard deviation of three replicates, while confidence intervals for predicted values are based on a 95 % confidence level.

Under optimal extraction conditions, the TPC value in this study is 77 % higher than the initial value of fresh OP, supporting the findings in Section 3.3, where temperature and extraction time in autohydrolysis enhanced the solubilization of biophenols. Rubio-Senent *et al.* (2012) reported a 93 % TPC increase after steam heat treatment at 160 °C for 75 min, while Gimenez *et al.* (2020) observed a two-fold increase using water extraction under harsher conditions (240 °C, 4 h, liquid-solid ratio: 1.7 mL/g). The TPC obtained in this study (25.65 ± 0.17 mg GAE/g) surpasses that reported by Rubio-Senent *et al.* (2012) (11.43 mg GAE/g) and is comparable to the work of Gimenez *et al.* (2020) (35.82 mg GAE/g), despite the fact that the extraction conditions differed. Dauber *et al.* (2022a) extracted biophenols from OP using supercritical CO_2 with ethanol as a co-solvent. Although their method differs, their OP source (Maldonado, Uruguay) and variety (Arbequina) offer a relevant comparison. Notably, this study's TPC values are significantly higher than that reported by Dauber *et al.* (2022a), who documented an optimal value of 0.073 mg GAE/g.

A direct comparison of the antioxidant activity of the obtained extracts with studies using autohydrolysis for biophenol extraction from OP or other olive oil by-products is challenging due to differing in vitro techniques (Herrero *et al.*, 2012; Rubio-Senent *et al.*, 2012). However, comparing results with studies using alternative extraction methods is insightful. Under optimal conditions, this study's results align well with those of Nunes *et al.* (2021), who extracted biophenols from OP using 80 % ethanol-water in a shaking water bath at room temperature for 3 h, reporting FRAP values of 111–233 μmol FSE/g and DPPH values of 50–85 μmol TRE/g. Similarly, this study compares favorably with Quero *et al.* (2022), who extracted OP using water and 50 % ethanol-water via ohmic heating and stirred water bath at 80 °C for 30 min (solid-liquid ratio: 0.1 g/mL), reporting FRAP values of 80 μmol FSE/g for water extracts and 130–150 μmol FSE/g for ethanol-water extracts.

Regarding the HTY content, the results obtained in the present study under optimal extraction conditions are comparable to those obtained by Lama-Muñoz *et al.* (2019), who used an autohydrolysis process applied to OP with steam at 160 °C and various extraction times (15-90 min). They reported maximum HTY content values of 3.8 mg/g. Likewise, Rubio-Senent *et al.* (2012) reported a HTY content of 5.3 mg/g under the extraction conditions previously mentioned. Gimenez *et al.* (2020) reported a higher HTY content (7.0 mg/g) compared to that obtained in the present study using autohydrolysis applied to OP, but under more severe treatment conditions (240 °C and 4 h).

With respect to the HMF content, dissimilar results have been published. Rubio-Senent *et al.* (2012) reported a maximum content (0.07 mg/g) significantly lower than that reported in the present study under optimal extraction conditions, applying autohydrolysis to OP using steam at 160 °C for 90 min. On the other hand, Herrero *et al.* (2012) reported a maximum HMF content of 49.5 mg/g for the case of extraction with subcritical water from olive leaves, under extraction conditions of 200 °C for 20 min at 1500 psi. Beyond the different values reported in the literature, it is interesting to know the evolution in the formation of HMF when applying extraction processes through autohydrolysis, as discussed in Section 3.4.

The biophenol content and antioxidant activity of OP extracts depend not only on extraction conditions and technologies but also on cultivation factors like location, variety, harvest time, and maturity stage (Dauber *et al.*, 2022a). In this regard, it is interesting to compare the present results to those previously reported by our group using solid-liquid extraction with natural deep eutectic solvents (NADES) (Cabrera *et al.*, 2024), as the same starting OP was used in both studies. The results of TPC, antioxidant activity and HTY content obtained in the present work under the optimal extraction conditions were significantly higher than those reported by Cabrera *et al.* (2024), who used lactic acid-glucose (5:1 molar ratio) with a water content of 68

% (w/w) as the extraction solvent, a temperature of 80 °C, an extraction time of 80 min, and a solid-liquid ratio of 0.014 g/mL (TPC: 15.56 mg GAE/g; FRAP: 178.14 μmol FSE/g; DPPH: 72.75 μmol TRE/g and HTY content: 1.24 mg/g). This underscores the effectiveness of the current extraction method using water as solvent for obtaining biophenols with antioxidant activity from OP in comparison with the use of NADES.

3.6. Oxidative stability evaluation

The effect of OP extract, BHA, and BHT on the oxidative stability of SFO was evaluated using the Rancimat equipment. Table 5 presents the induction period (IP) and relative protection factor for SFO supplemented with OP extract (500–5000 ppm), BHA (100 ppm), and BHT (200 ppm). IP served as an indicator of antioxidant stability, with higher values reflecting greater protection. Increasing OP extract concentration improved SFO stability. At 2500 ppm, the extract provided greater protection than BHA (relative protection factor: 1.7 vs. 1.6). At 5000 ppm, it outperformed BHT (2.1 vs. 1.8). These results demonstrate that OP extract, obtained under optimal conditions, offered superior oxidative stability compared to synthetic antioxidants. Similar findings were reported by Dauber *et al.* (2022a), where OP extract (Arbequina) enhanced soybean oil stability, achieving relative protection factors of 3.6 (2000 ppm) and 4.5 (5000 ppm). These results highlight OP extract's potential as a natural alternative to synthetic antioxidants in the food industry.

On the other hand, it is worth discussing the final HMF content in the food after adding the OP extract. Considering the EY and the HMF content in the OP extracts, as reported in Table 4, for the highest extract concentration used in the SFO (5000 ppm), the resulting HMF content would be 18 mg/kg of SFO. Interestingly, current regulations from the Codex Alimentarius, the European Union, and the United States set limits exclusively for honey, ranging from 40 to 80 mg/kg depending on the country of origin (Martins *et al.*, 2022). In the juice industry, the International Federation of Fruit Juice Processors recommends 5–10 mg/L of HMF in fruit juices (25 mg/kg for concentrates), while the European Union sets a 20 mg/kg limit for juices intended for children (Lee *et al.*, 2019). Therefore, although no legal limits exist for HMF in oils, given that daily consumption of these products is lower than that of fruit juices, the HMF amounts added in this study appear, in principle, to be within acceptable ranges. In any case, further analyses are needed to determine the effect of the spray drying conditions on the final HMF content in the extracts.

TABLE 5. Induction period obtained by the Rancimat method applied to SFO additivated with different concentrations of OP extract and with BHA and BHT

Sample	IP*	Relative protection factor§
SFO	7.3 ± 0.3^{a}	
SFO + 500 ppm extract	8.8 ± 0.1^{b}	1.2
SFO + 1000 ppm extract	9.3 ± 0.1^{c}	1.3
SFO + 2500 ppm extract	12.6 ± 0.2^{e}	1.7
SFO + 5000 ppm extract	15.4 ± 0.1^{g}	2.1
SFO + 200 ppm BHA	11.8 ± 0.2^{d}	1.6
SFO + 100 ppm BHT	13.2 ± 0.1^{f}	1.8

* Induction period (h). Values are expressed as the mean ± standard deviation of three replicates. Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).
§ Relative protection factor was calculated as: (IP of SFO + extract or antioxidant added)/(IP of SFO).

3.7. Antibiofilm activity

The OP extract effectively inhibited biofilm formation in *C. albicans* and *S. aureus* in a dose-dependent manner (Figure 3). At 5 mg/mL, inhibition exceeded 90 %, highlighting its potential for infection prevention. However, it had no effect on the Gram-negative bacterial biofilms tested. While OP extracts have shown antibacterial activity against planktonic bacteria, including *S. aureus* (Ferguous *et al.*, 2023; Khadim *et al.*, 2020), studies on

their antibiofilm properties are limited, particularly regarding polyphenols. Polyphenols are well-documented for their antibacterial and antibiofilm effects via multiple mechanisms (Slobodníková *et al.*, 2016). Given the need for multitarget strategies to reduce resistance development, whole extracts may be more effective than isolated compounds. The extract's strong antioxidant activity may contribute to its antibiofilm effect, as reactive oxygen species are believed to play a key role in biofilm formation (Gambino and Cappitelli, 2016).

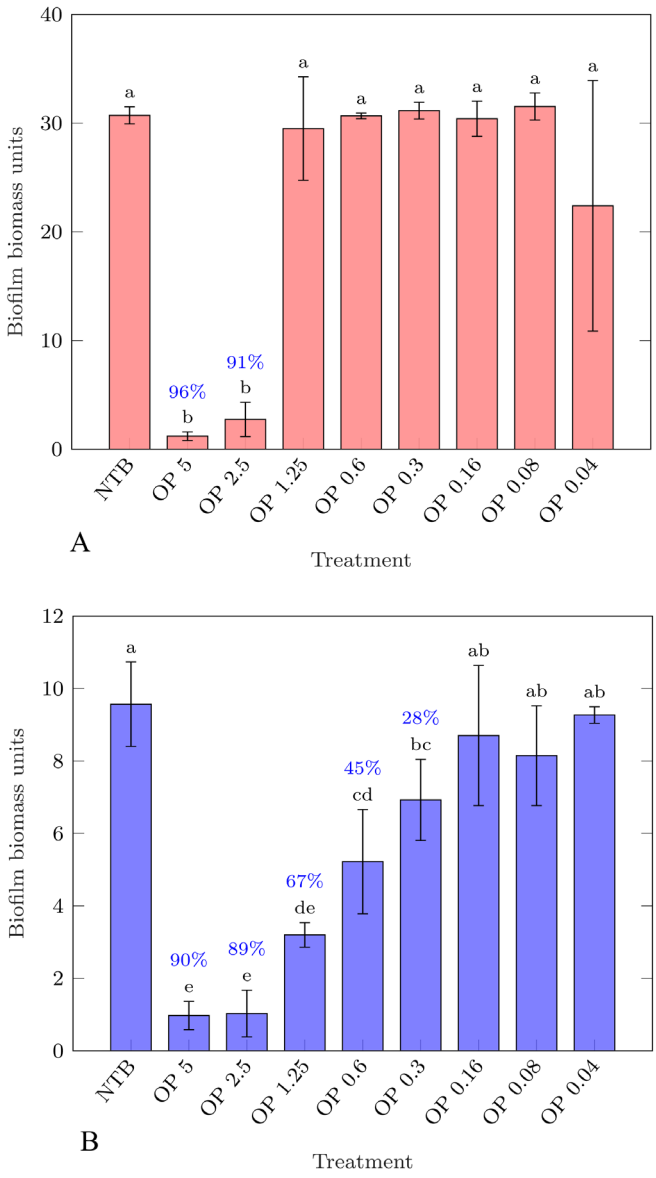

FIGURE 3. Inhibition of: A) *Candida albicans* biofilms and B) *Staphylococcus aureus* biofilms, by different concentrations (0.04-5 mg/mL) of OP extract. Values are expressed as the mean ± standard deviation of four replicates. Different letters represent significant differences (Tukey test, $p < 0.05$). Inhibition percentages are shown above each bar for treatments able to inhibit biofilm formation.

4. CONCLUSIONS

This study evaluated autohydrolysis as a green method for extracting biophenols with antioxidant and antibiofilm properties from Uruguayan olive pomace, using only water as the solvent while considering degradation compounds like HMF.

Extraction yield (EY), total phenolic content (TPC), antioxidant activity, hydroxytyrosol (HTY), and HMF content were significantly influenced by temperature, extraction time, and liquid-solid ratio. Mathematical

models were developed to assess both the benefits (maximizing phenolic recovery and antioxidant activity) and drawbacks (degradation compound formation). Optimal conditions (168 °C, 24 min, 20 mL/g) were identified to enhance EY, TPC, antioxidant activity, and HTY while minimizing HMF. Under these conditions, the extract showed excellent sunflower oil stabilization and effectively inhibited *Candida albicans* and *Staphylococcus aureus* biofilm formation.

These findings confirm autohydrolysis as an effective method for producing olive pomace extracts with potential food and pharmaceutical applications. Future research should explore process scalability, full chemical characterization of the extracts, purification techniques for higher extract purity, and the recovery of additional valuable compounds within a biorefinery framework for comprehensive by-product utilization.

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DECLARATION OF COMPETING INTEREST

The authors of this article declare that they have no financial, professional or personal conflicts of interest that could have inappropriately influenced this work.

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AUTHORSHIP CONTRIBUTION STATEMENT

L. Cabrera: Conceptualization, Methodology, Software, Formal analysis, Investigation, Funding acquisition, Writing – original draft, Writing – review & editing. **L. Xavier**: Conceptualization, Supervision, Project administration, Writing – review & editing. **I. Vieitez**: Formal analysis and investigation of section Oxidative stability, Writing – original draft of section Oxidative stability, Writing – review & editing. **S. Raffaelli**: Formal analysis and investigation of section Antibiofilm activity, Writing – original draft of section Antibiofilm activity, Writing – review & editing. **S. Alborés**: Formal analysis and investigation of section Antibiofilm activity, Writing – original draft of section Antibiofilm activity, Writing – review & editing. **B. Zecchi**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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