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Ultrasound-assisted extraction of hydroxytyrosol and tyrosol from olive pomace treated by gamma radiation: process optimization and bioactivity assessment

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Ultrasound-assisted extraction (UAE) was used to recover hydroxytyrosol and tyrosol from olive pomace, a residue generated by the olive oil industry. The extraction process was optimized using response surface methodology (RSM), with processing time, ethanol concentration and ultrasonic power as the combined independent variables. The highest amounts of hydroxytyrosol (36 ± 2 mg g^{-1} of extract) and tyrosol (14 ± 1 mg g^{-1} of extract) were obtained after 28 min of sonication at 490 W using 7.3% ethanol as the solvent. Under these global conditions, an extraction yield of 30 ± 2% was achieved. The bioactivity of the extract obtained under optimized UAE was evaluated and compared with that of an extract obtained under optimal heat-assisted extraction (HAE) conditions in a previous work of the authors. Compared to HAE, UAE reduced the extraction time and the solvent consumption, and also led to higher extraction yields (HAE yield was 13.7%). Despite this, HAE extract presented higher antioxidant, antidiabetic, anti-inflammatory and antibacterial activities and no antifungal potential against *C. albicans*. Furthermore, HAE extract also showed higher cytotoxic effects against the breast adenocarcinoma (MCF-7) cell line. These findings provide useful information for the food and pharmaceutical industries in developing new bioactive ingredients, which may represent a sustainable alternative to synthetic preservatives and/or additives.

1. Introduction

Olive pomace, although an environmentally harmful waste generated during olive oil extraction, contains considerable amounts of phenolic compounds such as hydroxytyrosol and tyrosol, oleuropein and its derivatives, phenolic acids and flavonoids[1–4] with biological properties that can have a positive impact on human health.[5–7] In recent years, the demand for natural antioxidants as safe alternatives to the synthetic ones has increased, in part due to the growing interest of consumers in healthier food. In this regard, olive wastes could be considered valuable sources of natural bioactive compounds to be added to foods as natural additives and supplements.

The development of efficient extraction processes to recover these compounds from olive pomace is an important challenge in order to obtain added-value products, maintaining the health and sustainability of the environment and industries. There are different methods for extracting phenolic compounds from olive pomace. The most used and simplest technique for this purpose is maceration; however, some emerging technologies have been developed to reduce the extraction time and the volume of solvents and to increase the extraction selectivity and purity.[8] These emerging technologies include superheated liquid extraction (SLE),[9] ultrasound-assisted extraction (UAE),[10,11] microwave-assisted extraction (MAE),[12] pressurized liquid extraction (PLE),[13] supercritical fluid extrac-

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tion (SFE)[14] and multi-frequency multimode modulated ultrasonic technique.[4] Among these techniques, UAE is considered the simplest and most cost-effective one to use at both laboratory and industrial scale, although the use of high power levels can lead to the degradation of some compounds.

Ionizing radiation is a clean and environmentally friendly technology, that has been proved to improve phenolic extraction from and the antioxidant activity of industrial wastewater,[15] fresh fruits such as cherry tomatoes,[16] raspberries[17,18] and strawberries,[19] and dried medicinal/aromatic plants.[20] More recently, the potential of 5 kGy gamma radiation as an enhancer for phenolic compound extraction and the antioxidant capacity of olive pomace extracts was demonstrated, increasing the extractability of the main phenolic compounds by 2.4-fold in comparison with non-irradiated samples.[2]

Response surface methodology (RSM) has been proposed to provide information regarding the optimal combination of extraction factors to obtain natural antioxidants from olive pomace, using different extraction methods, such as heat-assisted extraction (HAE),[21,22] PLE[13] and UAE,[10,11,23] In fact, Goldsmith *et al.* (2018)[10] demonstrated that UAE increased the extraction of total phenolic compounds by 24% and the HPLC peak areas by 20.4%, whereas the antioxidant activity was increased by 11%. The current study aims to explore the efficiency of UAE for extracting phenylethanoids from olive pomace using a circumscribed central composite design (CCCD) testing different conditions, namely the percentage of ethanol (0–100%), extraction times (2–40 min) and ultrasonic power (5–500 W). The olive pomace samples used in this work were irradiated ones for which the best results for improving bioactive extractability were obtained in a previous work of the authors.[2] The extraction yields of the individual hydroxytyrosol and tyrosol were obtained, summarized and modeled by RSM, in order to understand the combined effects of the operating variables and to maximize the responses analyzed. Furthermore, the biological properties (antioxidant, antimicrobial, antidiabetic and anti-inflammatory activities and cytotoxicity) of olive pomace extracts obtained under UAE optimized conditions were evaluated, and compared with those of the extracts obtained under optimized conditions.[21] As far as the authors know, this is the first time that a comprehensive study of the bioactivity has been performed comparing both processes for extracting bioactive compounds from olive pomace, in the hope that the results can contribute to the development of new ingredients to be used by the food industry.

2. Materials and methods

2.1. Olive pomace samples

The samples used in this work were olive pomaces collected in November 2020 from UCASUL (União de Cooperativas Agrícolas do Sul, located in the Alentejo region, Portugal) and further submitted to a gamma radiation treatment as described by Madureira *et al.* (2020).[2]

2.2. Irradiation experiments

Irradiation was carried out in a Co-60 semi-industrial unit (with an activity of 126 kCi in March 2021) located at the Technological Unit of Radiosterilization (UTR-IST), University of Lisbon (Portugal). Sealed bags (20 cm × 10 cm) containing 100 g of extracted olive pomace were irradiated at room temperature at 5.2 ± 0.2 kGy using a dose rate of 10.4 kGy h^{-1}. The absorbed doses were measured using Amber Perspex routine dosimeters[24] (dose uniformity DUR = 1.07). The irradiations were performed in triplicate.

Fig. 1 presents a schematic diagram of the different steps carried out for the optimization of the phenolic compound extraction from irradiated olive pomace.

2.3. Experimental design for extraction optimization

A five-level circumscribed central composite design (CCCD) coupled with RSM was implemented to optimize the extraction of phenylethanoids from olive pomace. The coded and natural values of the independent variables X_A (time: t, min), X_B (power: P, W), and X_C (solvent proportion: S, % ethanol, v/v) are presented in Table 1. The 28 experimental points of the

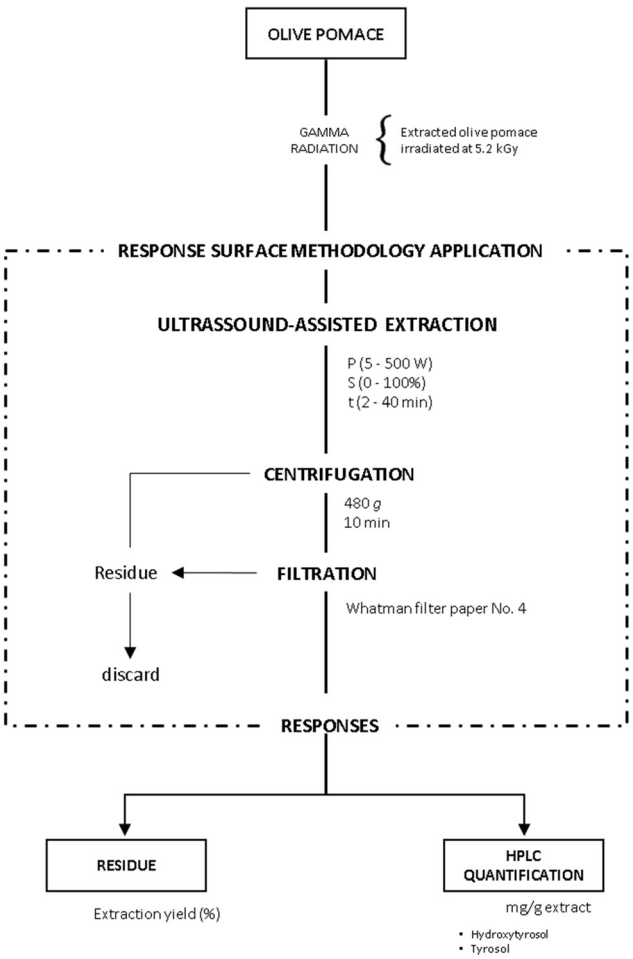

Fig. 1 Diagram of the different steps carried out for the extraction optimization of the phenolic compounds from irradiated olive pomace.

Table 1 Natural and coded values of the independent variables used in the 5-level central composite designs (CCD) implemented for optimization of ultrasound-assisted extraction using response surface methodology (RSM)

	Natural values		
Coded values (α)	Time X_A (min)	Power X_B (W)	Solvent proportion X_C (%, v/v)
−1.68	2	5	0
−1	9.7	105.3	20.3
0	21	252.5	50
1	32.3	399.7	79.7
1.68	40	500	100

CCCD design shown in Table 2 were generated using Design-Expert software, version 11 (Stat-Ease, Inc., Minneapolis, USA) by entering factor ranges in terms of alphas (α = −1.68, −1, 0, 1, 1.68), and included 6 replicates at the central point (three replicates per condition). The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

2.4. Ultrasound-assisted extraction (UAE)

All irradiated olive pomace samples were immediately lyophilized (Heto CD8, Allerod, Denmark) and stored in dark conditions at room temperature until used. The UAE was performed using an ultrasonic system (ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe and a connector for the sample temperature control, according to a methodology previously described by Albuquerque *et al.* (2020).[25] Olive pomace (0.9 g) was placed in 30 mL of solvent with different conditions previously defined by the RSM design (Table 1): time (t, 2–40 min), power (P, 5–500 W) and ethanol proportion (S, 0–100%). These experimental range conditions were selected from several screening analyses, based on experimental data previously obtained by the research group. The solid/liquid ratio was kept constant at 30 g L^{-1}, as well as temperature (T, 30–35 °C; a cold-water bath was used to control the temperature). After the extraction, the samples were centrifuged (480g for 10 min at room temperature) and filtered (paper filter; Whatman no. 4) and the supernatant was collected.

2.5. Analyzed responses

2.5.1. Extraction yield. The residue resulting from each extraction was determined gravimetrically using crucibles by the evaporation of 5 mL of supernatant (extract solution) at 100 °C for 24/48 h. The results are expressed as percentages (%, w/w).

2.5.2. Chromatographic analysis of phenolic compounds. A portion (1.5 mL) of each extract solution was filtered through 0.22 μm disposable syringe filters and analysed by HPLC–

Table 2 Experimental results obtained under the extraction conditions defined in the circumscribed central composite design (CCCD) matrix for extraction yield and contents of hydroxytyrosol (HYD), tyrosol (TYR) and both phenylethanoids (PhC) as a function of the extraction method. The natural values of the independent variables X_A (time), X_B (power) and X_C (solvent proportion) are presented in Table 1

	CCCD experimental design			Residue	Phenolic content by HPLC		
Run	X_A: t (min)	X_B: P (W)	X_C: S (%)	Yield (%)	HYD (mg g^{-1} ext)	TYR (mg g^{-1} ext)	PhC (mg g^{-1} ext)
1	9.7	105.3	20.3	13.15	28 ± 1	9.9 ± 0.5	37 ± 2
2	9.7	105.3	20.3	11.46	28 ± 1	10.1 ± 0.3	38 ± 1
3	32.3	105.3	20.3	15.53	27 ± 2	9.6 ± 0.5	36 ± 2
4	32.3	105.3	20.3	12.61	27 ± 1	9.7 ± 0.3	37 ± 1
5	9.7	399.7	20.3	22.30	33 ± 1	11.8 ± 0.3	44 ± 1
6	9.7	399.7	20.3	24.14	32 ± 2	11.8 ± 0.5	44 ± 2
7	32.3	399.7	20.3	24.86	33.2 ± 0.5	12.3 ± 0.3	45 ± 1
8	32.3	399.7	20.3	23.32	33 ± 1	12.4 ± 0.1	45 ± 1
9	9.7	105.3	79.7	2.91	22 ± 1	10.9 ± 0.2	32 ± 1
10	9.7	105.3	79.7	1.66	24 ± 1	12.2 ± 0.5	36 ± 2
11	32.3	105.3	79.7	5.85	16.6 ± 0.1	8.03 ± 0.22	24.7 ± 0.3
12	32.3	105.3	79.7	2.27	18.4 ± 0.1	9.20 ± 0.03	27.6 ± 0.2
13	9.7	399.7	79.7	13.54	14.8 ± 0.3	8.1 ± 0.2	22.9 ± 0.5
14	9.7	399.7	79.7	11.14	16.6 ± 0.3	8. ± 0.1	25.2 ± 0.2
15	32.3	399.7	79.7	17.51	18.2 ± 0.6	9.53 ± 0.01	28 ± 1
16	32.3	399.7	79.7	16.96	16.4 ± 0.3	8.7 ± 0.1	25.04 ± 0.19
17	2	252.5	50	3.85	54 ± 1	21.2 ± 0.1	75 ± 1
18	40	252.5	50	15.15	27.8 ± 0.3	10.4 ± 0.4	38 ± 1
19	21	5	50	5.05	29.7 ± 0.3	12.9 ± 0.4	43 ± 1
20	21	500	50	23.34	26.9 ± 0.2	10.3 ± 0.4	37.1 ± 0.2
21	21	252.5	0	21.12	34 ± 5	11 ± 2	46 ± 7
22	21	252.5	100	0.93	5.9 ± 0.4	4.5 ± 0.4	10.5 ± 0.8
23	21	252.5	50	16.96	25.8 ± 0.1	10.7 ± 0.1	36.5 0.2
24	21	252.5	50	12.26	28.6 ± 0.5	11.5 ± 0.3	40 ± 1
25	21	252.5	50	15.18	24.7 ± 0.3	9.9 ± 0.2	34.6 ± 0.1
26	21	252.5	50	12.50	25.7 ± 0.3	10.3 ± 0.4	35.94 ± 0.04
27	21	252.5	50	19.14	24.89 ± 0.04	10.9 ± 0.2	35.8 ± 0.3
28	21	252.5	50	14.56	25.35 ± 0.08	10.80 ± 0.05	36.15 ± 0.03

DAD–ESI/MS^n (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) using the conditions described by Bessada *et al.* (2016).[26] Quantitative analysis was performed from the peak areas recorded at 280 nm using 9-level calibration curves (1.25–80 μg mL^{-1}) obtained from commercial standards of hydroxytyrosol ($y = 124154x + 17\,393$, $R^2 = 0.999$, LOD (limit of detection) = 1.22 μg mL^{-1}; LOQ (limit of quantification) = 3.68 μg mL^{-1}) and tyrosol ($y = 91708x - 9398.5$, $R^2 = 0.999$, LOD = 0.91 μg mL^{-1}; LOQ = 2.77 μg mL^{-1}). The results are expressed as mg per g of extract, and the final responses processed for all compounds were summed up to calculate phenylethanoid content (PhC).

2.6. Extraction optimization by response surface methodology

2.6.1. Response criteria and mathematical modelling. The extraction yield and levels of hydroxytyrosol (HYD) and tyrosol (TYR), as well as the total amount resulting from the sum of both compounds (TPC), were the four response variables considered to optimize the recovery of phenolic compounds from olive pomace.

The response surface models were fitted to the second-order polynomial model (eqn (1)):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>1}}^{n=1} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the dependent (response) variable to be modelled; X_i and X_j are the independent variables; b_0 is a constant coefficient (intercept); b_i, b_{ii} and b_{ij} are the coefficients of the linear, quadratic, and interactive terms, respectively; and n is the number of tested variables.

2.6.2. Fitting procedures and statistical analysis. Fitting procedures, coefficient estimates and statistical calculations were performed using Design-Expert software, version 11. The analyses of variance (ANOVA) was used to determine the regression coefficients and to assess the significance of the data. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}), interpreted as the proportion of variability of the dependent variable explained by the model,[25] were used to estimate the fitness of the polynomial equation to the response. Only the statistically significant terms (p-value < 0.05) were used to fit the mathematical models. The significance of all the terms of the polynomial equations was analyzed statistically by computing the F-value at $p < 0.05$. The statistic lack of fit was used to evaluate the adequacy of the models. This test shows whether the model adequately describes the functional relationship between the independent variables and the obtained response. Thus, the lack of fit should be non-significant ($p > 0.05$). The software was also used to generate the response surface graphs.

2.7. Evaluation of the bioactive properties

The olive pomace extract obtained under the optimal conditions using UAE, as described in section 2.6, was used for evaluating the bioactive properties described below. This UAE-obtained extract was also compared with the olive pomace extract from the optimal conditions obtained using HAE.[21]

2.7.1. Antioxidant assessment. The antioxidant capacity of olive pomace was measured through the inhibition of lipid peroxidation by the decrease in thiobarbituric acid reactive substance (TBARS) formation and the oxidative hemolysis (OxHLIA) inhibition assays. Trolox was used as a positive control.

The TBARS assay measures the extract's capacity to inhibit the formation of malondialdehyde and other TBARS and was performed using porcine brain tissues as an oxidizable substrate, following the procedure previously described by Mandim *et al.* (2020).[27] The olive pomace extracts were re-dissolved in water to obtain a stock solution at 8 mg mL^{-1}, which was further diluted to obtain the range of concentrations tested (0.078–1 mg mL^{-1}). The results were expressed as the extract concentration (IC_{50}, μg mL^{-1}) that causes 50% inhibition of the oxidation process.

The OxHLIA assay measures the extract's capacity to inhibit the oxidative hemolysis and was performed using erythrocytes isolated from sheep blood collected from healthy animals, following the procedure previously described by Mandim *et al.* (2020).[27] The olive pomace extracts were re-dissolved in PBS to obtain a stock solution at 8 mg mL^{-1}, then diluted to achieve different solutions ranging from 0.078 to 0.25 mg mL^{-1}. The results were expressed as the extract concentration (IC_{50}, μg mL^{-1}) required to maintain the integrity of 50% of the erythrocyte population after 60 and 120 min.

2.7.2. Antimicrobial activity. The antimicrobial activity was assessed using three Gram-negative bacteria (*Escherichia coli* (ATCC 8739™), *Pseudomonas fluorescens* (ATCC 13525™) and *Salmonella enterica* serotype Typhimurium (ATCC 14028™)), three Gram-positive bacteria (*Staphylococcus aureus* (ATCC 6538™), *Bacillus cereus* (SSI C1/1) and *Listeria monocytogenes* (ATCC 19111™)) and a fungus (*Candida albicans* (ATCC 10231™)). The bacterial or fungal suspension was adjusted with sterile saline solution to a concentration of 1×10^5 CFU mL^{-1}. The extracts of olive pomace were dissolved in 10% DMSO in Tryptic Soy Broth (TSB) medium at a final concentration of 300 mg mL^{-1}. Then, several concentrations (12.5–100 mg mL^{-1}) were prepared directly in the well and 100 μL of the respective inoculum added (1×10^4 CFU/well). The minimum inhibitory (MIC) and minimum bactericidal (MBC) or minimum fungicidal (MFC) concentrations were determined by the microdilution method.[28] Streptomycin and ketoconazole were used as a positive control and 3% DMSO was used as negative control. Samples were tested in triplicate and each experiment was repeated three times.

2.7.3. Antidiabetic activity. The antidiabetic activity of olive pomace extracts was assessed using two different enzymes: α-amylase and α-glycosidase.

The α-amylase inhibitory activity of olive pomace extracts was measured following the method of Chen *et al.* (2020).[29] Firstly, 20.0 μL of olive pomace extracts with different concentrations (12.5–200 mg mL^{-1}, prepared in 0.1 M phosphate-

buffered saline (PBS, pH 6.9)) and 20.0 μL of α-amylase solution (1.0 U mL^{-1} in 0.1 M PBS) were mixed in a 1.5 mL centrifuge tube and incubated at 37 °C for 10 min. Then, 40.0 μL of starch solution (0.5%, w/v, in 0.1 M PBS) was added and reacted at 37 °C for another 10 min. After this, 80.0 μL of 3,5-dinitrosalicylic acid (DNS) reagent was added and the mixture was incubated in boiling water for 5 min to terminate the reaction. The absorbance was measured at 540 nm using a microplate reader (EZ Read 2000, Biochrom, Cambridge, UK). Acarbose was used as the positive control and individual blanks were prepared. The inhibition rate of α-amylase was calculated according to the following formula: α-amylase inhibition (%) = $[1 - (Abs_1 - Abs_2)/Abs_0] \times 100$, where Abs_0 is the absorbance of a mixture containing α-amylase and starch solution, Abs_1 was the absorbance of mixture containing α-amylase, starch solution and extract; and Abs_2 is the absorbance of a mixture containing extract and starch solution. The assay was performed in triplicate.

The capacity of olive pomace extracts to inhibit α-glycosidase was measured based on the methodology previously described by Silva *et al.* (2020).[30] The assay was conducted in a 96-well microplate with a reaction mixture containing 50 μL of olive pomace extract at different concentrations (3.125–100 mg mL^{-1}, prepared in 0.1 M phosphate-buffered saline (PBS, pH 6.9)) and 50 μL of yeast α-glucosidase (2 U mL^{-1} in 0.1 M PBS). The reaction mixture was then incubated for 10 min, followed by the addition of 50 μL of substrate (5 mM, *p*-nitrophenyl-α-D-glucopyranoside prepared in PBS). After 20 min of incubation at 37 °C, the release of *p*-nitrophenol was measured at 405 nm using a microplate reader (EZ Read 2000, Biochrom, Cambridge, UK). Individual blanks and a positive control (acarbose) were prepared. The results were expressed as IC_{50} values (mg mL^{-1}), which were calculated from the inhibition percentage values achieved using the formula: α-glycosidase inhibition (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$. The assay was performed in triplicate.

2.7.4. Cytotoxicity. The cytotoxic activity of olive pomace extracts (6.25–400 μg mL^{-1} in water) was evaluated using the sulforhodamine B assay, according to the method previously described.[31] Four human tumor cell lines (AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (lung carcinoma)) and two non-tumor cell lines (Vero (African green monkey kidney) and PLP2 (primary pig liver culture)) were used. Ellipticine was used as a positive control. The absorbance was read at 540 nm in the Biotek ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) and the results were expressed in terms of the concentration of the extract with the ability to inhibit cell growth by 50% – GI_{50} (μg mL^{-1}).

2.7.5. Anti-inflammatory activity. The capacity of olive pomace extracts (6.25–400 μg mL^{-1} in water) to inhibit the lipopolysaccharide (LPS)-induced nitric oxide (NO) production by a murine macrophage cell line (RAW 264.7) was assessed as the nitrite concentration in the culture medium.[32] The nitric oxide produced was determined by reading the absorbance at 540 nm (ELX800 Biotek microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). Dexamethasone was used as a positive control. The results were expressed in terms of the concentration of each extract that caused 50% inhibition of NO production – IC_{50} (μg mL^{-1}).

2.7.6. Statistical analysis. Data were expressed as mean ± standard deviation. In data analysis, confidence intervals for mean values were estimated considering a significance level of $p < 0.05$ and the number of replicates for each assay. The differences among treatments were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$.

3. Results and discussion

A previous study performed by the authors identified hydroxytyrosol and tyrosol as the most abundant phenolic compounds present in olive pomace.[2] It was also verified that gamma radiation at 5 kGy increased the extractability of bioactive compounds from olive pomace by 2.4-fold compared to the non-irradiated ones. Based on this information, the extraction of phenolic compounds from 5 kGy irradiated olive pomace by UAE was carried out by applying a RSM of three variables (*t*, *P* and *S*) in a *CCCD* with five levels of values for each variable. This multivariable approach could provide a consistent tool that minimizes experimental errors with a reduced number of tests, while optimizing the extraction conditions of the variables, according to the mathematical empirical models that predict the maximum extraction performance. After the determination of the optimal conditions of the analyzed variables, the bioactive properties of the extract were studied.

3.1. Optimization of the extraction of hydroxytyrosol and tyrosol

3.1.1. Experimental data obtained with *CCCD* design. The experimental results obtained with 28 runs of the five-level *CCCD* design matrix implemented to optimize the UAE to extract hydroxytyrosol and tyrosol from olive pomace are shown in Table 2. The extraction yield ranged from 0.93 to 24.86%. The lowest extraction yield was obtained with run 22, which combined medium *t* and *P* conditions (21 min and 252.5 W; $\alpha = 0$) with a high solvent concentration (100% ethanol, v/v; $\alpha = 1.68$), while the highest extraction yield was found with run 7, combining high *t* and *P* conditions (32.2 min and 399.7 W; $\alpha = 1$) and a low solvent concentration (20.3% ethanol, $\alpha = -1$). Concerning the phenolic compounds, hydroxytyrosol (HYD) was detected in higher quantities than tyrosol (TYR) in all the extracts, with concentrations ranging from 5.9 ± 0.4 to 54 ± 1 mg g^{-1} extract, while the TYR levels ranged from 4.5 ± 0.4 to 21.2 ± 0.1 mg g^{-1} extract. Furthermore, the highest levels of phenylethanoids (PhC; 75 ± 1 mg g^{-1} extract) were achieved with run 17, which employed lower *t* (2 min; $\alpha = -1.68$) and medium *P* (252.5 W; $\alpha = 0$) and *S* proportion (50% ethanol, v/v; $\alpha = 0$). As for extraction yield responses, higher ethanol concentrations promoted a negative effect on phenolic compound extraction by the UAE method,

as only 10.5 ± 0.8 mg PhC per g extract were obtained with run 22 (Table 2).

3.1.2. Analysis of the theoretical response surface models. RSM is a mathematical and statistical analysis tool used for optimizing processes involving one or more response variables. To elucidate how precisely the RSM can predict ideal variances is imperative to fit the models for the selected responses. In this work, the response values of Table 2 were fitted to the second-order polynomial model of eqn (1) using a non-linear least-squares procedure to develop the mathematical models (eqn (2)–(5)) and obtain the parametric values for each response criterion. The coefficients whose confidence interval value ($\alpha = 0.05$) was higher than the value of the parameter were considered statistically non-significant (Table 3) and, for this reason, were not used for the model development. The resulting models are presented below.

$$\mathrm{HYD} = 24.8800 + 0.0245P + 0.1494S - 0.0004PS - 0.0026S^2 \quad (2)$$

$$\mathrm{TYR} = 10.24513 - 0.0865t + 0.0044P + 0.0266S + 0.0003tP - 0.0002PS \quad (3)$$

$$\mathrm{PhC} = 41.5721 - 0.2772t + 0.0209P - 0.0392S + 0.0010tP - 0.0007PS \quad (4)$$

$$\mathrm{Yield} = 10.422 + 0.1209t + 0.0364P - 0.1654S \quad (5)$$

where P is ultrasonic power in W, t is the processing time in minutes and S is the ethanol concentration in % (v/v).

In mathematical terms, the sign of the parametric values determines the performance of the response: when the parametric value is positive, the response is higher at high levels whereas when it is negative, the response is lower at high levels. The higher the parametric value (in absolute terms), the more significant the weight of the corresponding variable is. The variables could be sorted in a descending order as a function of their significance in the extraction process as $S > P > t$. It was possible to observe (Table 3) that the evaluated responses were significantly affected by a linear effect, except for the variable t in HYD. Regarding the interactions between the three variables, it seemed that the more influential interactions occurred for $t \times P$ and $P \times S$, while $t \times S$ was only significant in PhC. In fact, yield was not affected by any of the variables, HYD was affected by $P \times S$, TYR by $t \times P$ and $P \times S$, and PhC by $t \times P$ and $t \times S$.

In addition, the obtained coefficients R^2_{adj} were 0.91, 0.52 and 0.88 for HYD, TYR and TPC, respectively (Table 3), indicating that the variability of each response could be explained satisfactorily for HYD and PhC and moderately for TYR by the independent variables involved in the process. All models presented a non-significant lack of fit (Table 3) ($p > 0.05$), demonstrating that the equations adequately described the effects of the variables on the evaluated responses.

In these studies, sometimes it can be difficult to not force the models to work with all the terms. To counter this situation, it is mandatory to monitor and adjust the model to significant terms or terms that display interactions, and control the statistical diagnostics. Furthermore, the compounds that are being extracted do not behave in the same way and it can be important to change the modeling within responses.

3.1.3. Effect of the independent variables on the target responses. The best way to visually describe and interpret all the effects and extraction trends is to generate 3D response surface plots that could represent the combined effects of two variables while keeping the other variable constant (Fig. 2).

The increase in the ethanol concentration affected negatively all the analyzed responses (Fig. 2), which indicated that the highest extraction of HYD and TYR was achieved for the

Table 3 Parametric results of the second-order polynomial equations for the four response value formats in terms of coded values. The parametric subscripts A, B, and C stand for the variables involving time (t), power (P) and solvent (S), respectively. Statistical analysis of the models is presented

Parameters		Yield	HYD	TYR	PhC
Effect		% (w/w)	mg g^{-1} extract	mg g^{-1} extract	mg g^{-1} extract
Intercept	b_o	13.89 ± 0.78	26.33 ± 0.58	10.12 ± 0.40	35.10 ± 1.01
Linear	b_A	1.37 ± 8.78	ns	0.03 ± 0.44	−0.36 ± 2.57
	b_B	5.36 ± 8.78	0.35 ± 0.74	0.20 ± 0.47	−0.76 ± 1.16
	b_C	−4.92 ± 8.77	−6.76 ± 0.94	−0.77 ± 0.47	−6.58 ± 1.15
Quadratic	b_{AA}	ns	ns	ns	ns
	b_{BB}	ns	ns	ns	ns
	b_{CC}	ns	−2.34 ± 1.11	ns	
Interaction	b_{AB}	ns	ns	0.58 ± 0.48	1.61 ± 1.22
	b_{AC}	ns	ns	ns	−3.16 ± 1.25
	b_{BC}	ns	−1.94 ± 1.1	−0.91 ± 0.51	ns
Statistical analysis	SM	<0.0001	<0.0001	0.0011	<0.0001
	LF	0.3966	0.0586	0.1391	0.0540
	R^2	0.8937	0.9165	0.6134	0.9041
	R^2_{adj}	0.8804	0.9125	0.5168	0.8789

HYD: hydroxytyrosol; TYR: tyrosol; PhC: phenylethanoids; SM: significance of the model; LF: lack of fit; R^2: coefficient of determination; R^2_{ajd}: adjusted coefficient of determination (R^2); ns: non-significant.

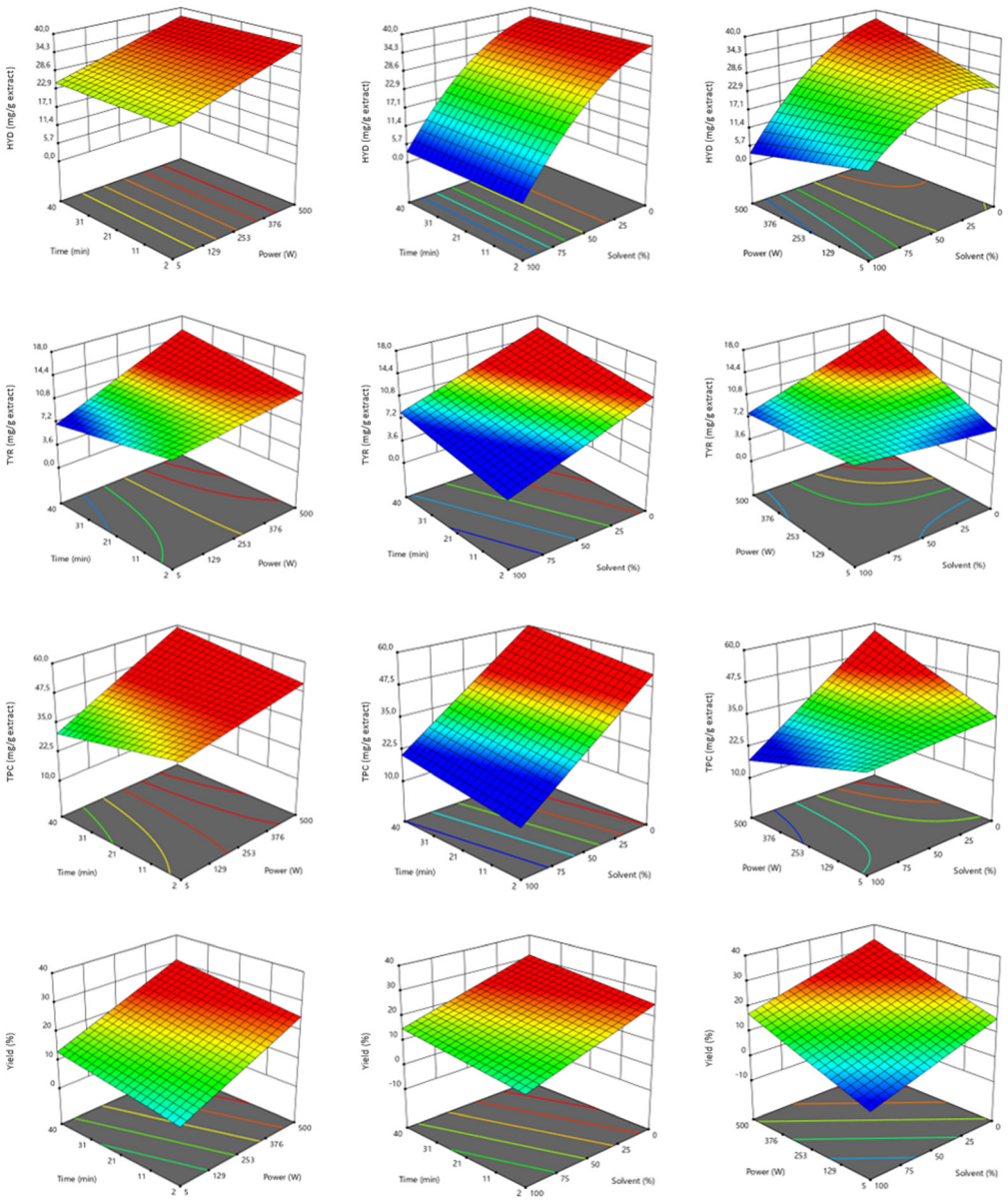

Fig. 2 Response surface graphs of the effect of the three independent extraction variables tested on the four evaluated responses: hydroxytyrosol (HYD), tyrosol (TYR) and phenylethanoids (PhC) contents and extraction yield (%). In each graph, the excluded variable was positioned at its optimal value (Table 4).

lowest ethanol concentrations. Furthermore, the extraction yield was also influenced by the ultrasound power and extraction time. The higher the ultrasound power and the extraction time, the higher the extraction yield, as can be confirmed by the positive sign obtained for these variables in contrast with the negative sign for the solvent (*i.e.* ethanol percentage) (Table 3). The response HYD was not significantly affected by the extraction time but it was favored by higher ultrasound power and lower ethanol concentrations. Both TYR and TPC responses were favored by higher ultrasound power and extraction time, and lower ethanol concentration. For these responses, a positive interaction between the two variables ($t \times P$) was also verified (Table 3). All these results supported the use of RSM as an optimization tool for the UAE optimization.

3.1.4. Optimal extraction conditions for maximizing the response criteria. After the statistical validation of the models, the optimal extraction conditions that maximize the individual and global responses were determined (Table 4).

In general, the best efficiency of the UAE process was obtained at short to medium extraction times, high ultrasound power, and low solvent percentages. Regarding the individual optimal conditions, it was possible to recover 37 ± 2 mg of hydroxytyrosol in 1 g of extract at 499 W and 13.6 min using 0% ethanol, while 15 ± 1 mg of tyrosol was obtained in 1 g of extract when applying the same ultrasound power (499 W) for

Table 4 Optimal individual and global conditions in natural values that maximize the extraction of phenolic compounds from olive pomace

Responses	Individual optimal conditions			Predictive responses	Global optimal conditions			Predictive response	Experimental responses
	t (min)	P (W)	S (%)		t (min)	P (W)	S (%)		
HYD (mg g^{-1} extract)	13.6	499	0.0	37 ± 2	28	490	7.3	36 ± 2	26 ± 1
TYR (mg g^{-1} extract)	36.4	499	3.8	15 ± 1				14 ± 1	9.7 ± 0.5
PhC (mg g^{-1} extract)	24.5	500	2.9	51 ± 3				50 ± 3	36 ± 2
Yield (%)	38.0	431	18.8	28 ± 2				30 ± 2	24 ± 1

HYD: hydroxytyrosol; TYR: tyrosol; PhC: phenylethanoids.

36.4 min and 3.8% ethanol. Similar individual optimal conditions (500 W for 24.5 min using 2.9% ethanol) were obtained for the recovery of 51 ± 3 mg of PhC in 1 g extract.

The global processing conditions that allowed simultaneous maximization of all the responses were also calculated (Table 4). The results demonstrated that 28 min of sonication at 490 W using 7.3% ethanol as the solvent were found to be the optimal conditions to recover 36 ± 2 mg of hydroxytyrosol per g of extract and 14 ± 1 mg of tyrosol per g of extract, achieving an extraction yield of 30 ± 2%.

It is important to mention that the comparison of the results from different studies can be difficult due to the variability of olive pomace samples (*e.g.*, the type of cultivation and the variety and maturation of the olives) and the different methods that are used to quantify the phenolic constituents. Furthermore, most studies in the literature did not evaluate the compounds individually, but in terms of total phenolic content and antioxidant activity. Martínez-Patiño *et al.* (2019)[11] and Gómez-Cruz *et al.* (2021)[33] selected slightly lower extraction times (15 and 12 min, respectively) to recover bioactive compounds from industrial exhausted olive pomace, although those authors used higher concentrations of ethanol (43.2%)[11] and acetone (40%).[33] On the contrary, Goldsmith *et al.* (2018)[10] optimized the extraction of phenolic compounds from olive pomace using only water as a solvent and a lower ultrasound power (250 W) than that obtained in this work, but higher extraction times (75 min). In terms of individual compounds, Xie *et al.* (2019)[12] recovered 55.1 ± 2.1 mg of hydroxytyrosol per g of olive pomace dry matter using 90% ethanol for 3 min, with an ultrasound power of 500 W. The amount of hydroxytyrosol obtained by Xie *et al.* (2019)[12] was higher than that found in the present study (10.2 ± 0.4 mg of hydroxytyrosol per g of dry weight – data not shown). Albahari *et al.* (2018)[23] reported that the addition of cyclodextrins to the extraction solvent under optimized UAE conditions enhanced the extraction of hydroxytyrosol from olive pomace when compared to the conventional methods and regular UAE, although the amounts of hydroxytyrosol obtained (523 ± 6–887 ± 13 mg kg^{-1} of olive pomace extract) were lower than the ones in the present work.

3.1.5. Experimental validation of the optimum extraction conditions. The global optimal conditions that maximize both the extraction yield and the recovery of phenolic compounds from olive pomace were then experimentally tested to analyze the model's ability to predict the experimental results (Table 4) and to obtain the phenolic-rich extracts that were used to evaluate the bioactive properties. UAE yielded 24 ± 1% and 36 ± 2 mg of PhC per g of extract, which means that the values differed by 20–28% from the predicted ones, so that the predictive capacity of the model could be considered acceptable and validated.

3.2. Evaluation of the bioactive properties

Once the optimal conditions of the analyzed variables were determined for UAE, the bioactive properties of the extract were evaluated. In a previous work, Madureira *et al.* (2021)[21] optimized the extraction of the same phenolic compounds from olive pomace using HAE and reported the highest extraction for longer extraction times (120 min), a temperature of 85 °C and 76% ethanol in water as the solvent. Comparing both techniques, UAE reduced the extraction time and the solvent consumption. Additionally, this method achieved higher extraction yields (the HAE yield was 13.7%), probably due to the structural changes in the solid sample promoted by the cavitation effects that could enhance the mass transfer. In order to determine the best conditions to obtain the extract with higher bioactivity, the extracts from the optimal conditions of both techniques (UAE and HAE) were compared in terms of antioxidant, antimicrobial, antidiabetic and anti-inflammatory capacities and cytotoxicity.

3.2.1. Antioxidant activity. Two methods were employed to evaluate the antioxidant activity of the extracts of olive pomace, OxHLIA and TBARS. The results are expressed as IC_{50} values (Table 5), meaning the extract concentration able to provide 50% of antioxidant activity (TBARS assay) or to protect 50% of the erythrocyte population from hemolysis caused by an oxidizing agent (OxHLIA assay). The IC_{50} values in the OxHLIA assay were obtained at two times (Δt 60 min and Δt 120 min) considering that natural extracts contain different antioxidant molecules capable of interacting with each other and offering protection at different time periods. In both assays, the lower the IC_{50} values, the higher the antioxidant capacity of the olive pomace extracts.

In the OxHLIA assay, the hemolysis curves for the olive pomace extracts of HAE and UAE at different concentrations (data not shown) revealed that higher concentrations protected the erythrocyte population from hemolysis for a longer period of time. The HAE extracts showed significant higher anti-

Table 5 Bioactive properties of extracts obtained under optimized HAE and UAE from olive pomace irradiated at 5 kGy

	Optimal conditions		
	HAE	UAE	Positive control
Extractability (mg g^{-1} ext)			
HYD	35.4 ± 0.6[a]	26 ± 1[b]	
TYR	13.8 ± 0.3[a]	9.7 ± 0.5[b]	
Cytotoxicity activity (GI_{50}, μg mL^{-1})			Ellipticine
AGS	238 ± 7[a]	230 ± 11[a]	1.23 ± 0.03[b]
CaCo	163 ± 12[a]	191 ± 10[a]	1.21 ± 0.02[b]
MCF-7	130.4 ± 10.5[b]	249 ± 17[a]	1.02 ± 0.02[c]
NCI-H460	234 ± 24[a]	226 ± 11[a]	1.01 ± 0.01[b]
PLP2	77 ± 4[a]	89 ± 8[a]	1.4 ± 0.1[b]
VERO	184 ± 9[a]	201 ± 20[a]	1.41 ± 0.06[b]
Anti-inflammatory activity (IC_{50}, μg mL^{-1})			Dexametasone
RAW 264.7	23 ± 1[b]	168 ± 6[a]	6.3 ± 0.4[c]
Antioxidant activity (IC_{50}, μg mL^{-1})			Trolox
TBARS inhibition	99 ± 5[b]	130 ± 5[a]	5.4 ± 0.3[c]
OxHLIA (Δ*t* 60 min)	18.5 ± 0.5[c]	45 ± 1[a]	21.8 ± 0.3[b]
(Δ*t* 120 min)	69 ± 3[b]	96 ± 2[a]	43.5 ± 0.8[c]
Antidiabetic activity (IC_{50}, mg mL^{-1})			Acarbose
α-Amylase inhibition	53 ± 10[b]	105 ± 11[a]	0.010 ± 0.003[c]
α-Glucosidase inhibition	14 ± 1[b]	26.1 ± 0.2[a]	11 ± 1[b]

HYD: hydroxytyrosol; TYR: tyrosol. In each row, different lowercase letters mean significant differences between average values ($p < 0.05$).

hemolytic activity (IC_{50} values of 18.5 ± 0.5 μg mL^{-1} and 69 ± 3 μg mL^{-1} at 60 and 120 min, respectively) than UAE extracts (IC_{50} values of 45 ± 1 and 96 ± 2 μg mL^{-1}) (Table 5). Furthermore, at 60 min, the IC_{50} value of HAE extract provided higher protection to the erythrocyte membranes than the positive control used, Trolox (IC_{50} value of 21.8 ± 0.3 μg mL^{-1}), demonstrating the significant antioxidant capacity of HAE extracts in the prevention of cellular oxidative processes. Nonetheless, a similar protection of the erythrocyte population (IC_{50} value of 20.6 ± 0.4 μg mL^{-1} at 60 min) in extracted olive pomace samples irradiated at 5 kGy was obtained using maceration extraction,[2] which is the same absorbed dose as applied in this work.

The HAE extracts were also more efficient in inhibiting the formation of TBARS than UAE extracts, with IC_{50} values of 99 ± 5 μg mL^{-1} and 130 ± 5 μg mL^{-1}, respectively (Table 5). Nevertheless, Trolox presented lower IC_{50} value (5.4 ± 0.3 μg mL^{-1}) than both the analyzed extracts, which could be attributed to the difference between a pure compound and the complex mixtures of olive pomace extracts.

The antioxidant activity of olive pomace extracts has usually been measured by chemical-based assays, namely for their DPPH and ABTS radical scavenging activity and ferric (FRAP) and cupric (CUPRAC) reducing power,[10,11,33–36] always obtaining the good correlation with the phenolic contents of olive pomace extracts.

3.2.2. Antimicrobial activity. The two extracts (HAE and UAE) of olive pomace were evaluated for their antimicrobial potential against three Gram-negative (*E. coli*, *S. typhimurium*, *P. fluorescens*) and three Gram-positive bacteria (*B. cereus*, *S. aureus*, *L. monocytogenes*) and one fungus (*C. albicans*).

The results showed that HAE extracts had higher antibacterial potential than UAE extracts, except for *S. typhimurium* (Table 6), which might be attributed to the higher amounts of hydroxytyrosol (35.4 ± 0.6 mg g^{-1} of extract) in HAE extracts (Table 5) that were proved to be effective against some pathogenic strains.[37] Moreover, concerning HAE extracts, Gram positive bacteria (MIC 25 mg mL^{-1}) seemed to be more sensitive than Gram negative bacteria (with the exception of *P. fluorescens*) (MIC 50 mg mL^{-1}), suggesting that the extracts could act differently on the cell wall of both bacterial types. Additionally, it is important to refer to the ability of both olive pomace extracts to inhibit the formation of *P. fluorescens* biofilm.

Regarding the antifungal potential, none of the extracts were demonstrated to have activity against *C. albicans* at the

Table 6 Antimicrobial activity of the extracts obtained under optimized HAE and UAE from olive pomace irradiated at 5 kGy

		B. cereus	*S. aureus*	*L. monocytogenes*	*E. coli*	*S. typhymurium*	*P. fluorescens*	*C. albicans*
		MIC (mg mL^{-1})						
Optimal conditions	HAE	25	25	25	50	50	25	>100
	UAE	50	50	50	100	50	50	>100
		MBC (mg mL^{-1})						MFC (mg mL^{-1})
Optimal conditions	HAE	25	100	100	100	100	25	>100
	UAE	50	>100	100	100	50	50	>100

studied concentrations (MIC > 100 mg mL^{-1}), which was also reported by Sousa *et al.* (2006)[38] in table olive extracts.

The antimicrobial potential of extracts obtained by maceration in 80% methanol from irradiated olive pomace samples was previously reported by the authors,[2] and there are also some works describing the antimicrobial activity of polyphenol extracts from olive oil,[39] olive cake[34] and olive leaves.[34,40,41] Interestingly, Moudache *et al.* (2020)[34] concluded that the extracts from olive leaves had higher antimicrobial activity than the extracts from olive cake against *S. aureus* (MIC 0.5 mg mL^{-1} and 8 mg mL^{-1} for olive leaves and olive cake, respectively) and *B. cereus* (MIC 1 mg mL^{-1} and 4 mg mL^{-1} for olive leaves and olive cake, respectively). On the other hand, in olive leaf extracts, Sweedan *et al.* (2019)[40] found MIC values of 7.5 mg mL^{-1} and 15 mg mL^{-1} against *S. aureus* and *E. coli*, respectively, while Gökmen *et al.* (2014)[41] reported MIC ≥ 32 mg mL^{-1} for *L. monocytogenes* and MIC ≥ 16 mg mL^{-1} against *B. cereus*, *S. aureus*, *E. coli* and *S. typhimurium*.

The obtained results demonstrated the potential of using irradiated olive pomace extracts as a food preservative in order to prevent the growth of foodborne pathogens together with their proved antioxidant properties.

3.2.3. Antidiabetic activity. Diabetes mellitus is caused by complete or partial deficiencies in insulin production/action and associated with various diseases such as hyperglycemia, hypertension, hyperlipidemia and cardiovascular diseases. Despite the traditional insulin therapy, demand by patients to use natural products to treat and prevent diabetes has been growing. The ability to inhibit α-amylase and α-glucosidase was reported to be effective to prevent type 2 diabetes, being useful to manage hyperglycemia.

The effectiveness of the HAE and UAE extracts in inhibiting α-amylase and α-glucosidase was expressed as IC_{50} values (Table 5). The HAE extracts presented significantly higher α-amylase (IC_{50} value of 53 ± 10 mg mL^{-1}) and α-glucosidase (IC_{50} value of 14 ± 1 mg mL^{-1}) inhibition activities than UAE extracts (IC_{50} values of 105 ± 11 and 26.1 ± 0.2 mg mL^{-1}, respectively). Furthermore, the α-glucosidase inhibition of HAE extracts (IC_{50} value of 14 ± 1 mg mL^{-1}) did not differ significantly from that for acarbose (IC_{50} value of 11 ± 1 mg mL^{-1}), the standard positive control.

As far as the authors know, this is the first study about the antidiabetic effect of olive pomace extracts, although the inhibitory activity of hydroxytyrosol and other phenolic compounds present in olive leaves and olive mill wastes was also demonstrated by Mwakalukwa *et al.* (2020)[42] and Hadrich *et al.* (2015).[43] On the other hand, extra virgin olive oils were reported to show a high digestive enzyme inhibitory activity that was associated with their phenolic composition.[44] Those authors also observed that olive oil samples with greater antioxidant effect were the most effective inhibitors of α-glucosidase.

The obtained findings revealed the existence of a relevant hypoglycemic effect, which could suggest the potential use of both extracts (and especially HAE extracts) as natural and promising antidiabetic agents.

3.2.4. Cytotoxicity and anti-inflammatory activity. The inhibitory effect of olive pomace extracts against the inflammatory response was measured using a cell-based screening bioassay to test the NO level in LPS-stimulated RAW 264.7 macrophages. Since NO has an important role in the inflammatory process, it is important to develop active formulations with natural compounds capable of inhibiting NO production. Both tested extracts showed anti-inflammatory capacity with IC_{50} values between 23 ± 1 and 168 ± 6 μg mL^{-1}, with HAE extracts presenting a much higher inhibitory activity in NO production (IC_{50} value of 23 ± 1 μg mL^{-1}) (Table 5).

There are no studies in the literature reporting the anti-inflammatory activity of extracts from irradiated olive pomace through this cell-based assay, but extra virgin olive oil was proved to have anti-inflammatory potential which was concentration dependent.[45]

The cytotoxic activity of the olive pomace extracts against four human tumor cell lines and two non-tumor cell lines was also tested. Both HAE and UAE extracts demonstrated cytotoxic activity for all the analyzed cell lines (Table 5). In absolute terms, the cell line CaCo-2 was the most susceptible to olive pomace extracts, followed by MCF-7, NCI-H460 and AGS (Table 5). Furthermore, there were no significant differences between the cytotoxic effects of both extracts, except for the breast adenocarcinoma (MCF-7) cell line, for which HAE extracts presented the highest activity (GI_{50} value of 130.4 ± 10.5 μg mL^{-1}) compared with UAE extracts (GI_{50} value of 249 ± 17 μg mL^{-1}).

The cytotoxicity of olive pomace methanol extracts against glioblastoma, breast, ovarian and pancreatic cancer cells was previously demonstrated by Goldsmith *et al.* (2018),[46] while Tezcan *et al.* (2017)[47] also showed that olive leaf extracts displayed toxicity towards glioblastoma cells.

4. Conclusions

The ultrasound-assisted extraction of phenolic compounds from olive pomace was evaluated using RSM. The results demonstrated that 28 min of sonication at 490 W using 7.3% ethanol as solvent were the conditions that maximized the extraction of hydroxytyrosol (36 ± 2 mg g^{-1} of extract) and tyrosol (14 ± 1 mg g^{-1} of extract), with a yield of 30 ± 2%. The bioactive properties of the extracts produced under the selected UAE optimal processing conditions were assessed and compared with the ones obtained using HAE. The UAE extracts presented lower bioactivity than the ones obtained by HAE. Despite reduced extraction time and solvent consumption by UAE as well as improved extraction yields in relation to HAE (yield of 13.7%), the HAE extracts showed greater antioxidant capacity, with lower extract concentrations being required to inhibit the TBARS formation and oxidative hemolysis, and also presented higher antibacterial and anti-inflammatory activities. Besides, the HAE extracts also presented higher antidiabetic potential and cytotoxic effect for the breast adenocarcinoma (MCF-7) cell line than those obtained by UAE.

According to these results, the HAE methodology was indicated to promote higher extractability and bioactivity of polyphenols from olive pomace compared with the UAE method.

The overall results in this work support the interest of food industries in developing new ingredients with bioactive potential from agro-industrial wastes, as natural alternatives to synthetic preservatives and/or additives, contributing to the sustainability of both the agro-industrial sector and the environment.

Author contributions

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Conflicts of interest

The authors declare that they have no conflict of interest.

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