Journal of Chromatography Open 6 (2024) 100161

Contents lists available at ScienceDirect

Journal of Chromatography Open

journal homepage: www.elsevier.com/locate/jcoa

Green extraction, chemical profile and biological activity of waste products from the olive oil industry: From waste to wealth

Miryam Perrucci [a,b], Marco Dezio [c], Hammad Saleem [d], Fabrizio Ruggieri [c], Marcello Locatelli [e,*]

[a] University of Teramo, Department of Biosciences and Agro-Food and Environmental Technologies, Teramo, 64100, Italy
[b] Department of Innovative Technologies in Medicine & Dentistry, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy
[c] Department of Physical and Chemical Sciences, University of L'Aquila, Via Vetoio, L'Aquila, Coppito, 67100, Italy
[d] Institute of Pharmaceutical Sciences (IPS), University of Veterinary & Animal Sciences (UVAS), Lahore, Pakistan
[e] Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy

ARTICLE INFO

Keywords:
Green chemistry
Green analytical chemistry
Green sample preparation
Circular economy
Olive oil
Chemical profile

ABSTRACT

Olive oil is the most used vegetable oil for human consumption and its production represents an important economic sector, especially in Mediterranean countries. Olive trees are grown in more than 40 countries around the world on over 10 million hectares. The milling industry generates large quantities of liquid and solid residues, the disposal of which requires sophisticated and rather expensive procedures, given the polluting characteristics of the processing products. Since a considerable measure of olive-derived biomass is generated each year, it could be used as a potential source of bioactive compounds. This work evaluates the possibility of recovering natural antioxidants from by-products of the olive oil mill, through the optimization of extraction processes with green approaches. In the present work, through HPLC-PDA analysis with a validated method, it was possible to characterize a chemical profile of the extracts obtained through an optimized (DoE) and green approach. The waste products of the olive oil companies represent the samples considered in this work, and are derived from the pomace and the washing water of 2-phases, 2.5-phases, and 3-phase extra virgin olive oil (EVO) production plants. The optimized extraction methodology, starting from the 2.5-phase olive pomace, proved to be satisfactory in terms of efficiency by evaluating the effect of parameters such as extraction time and process temperature. The application of this methodology to other types of pomace and agro-industrial by-products has highlighted excellent results in terms of extraction yield, demonstrating the validity of this procedure as also suitable for other solid residues coming from the olive oil mill. Regarding the treatment in vegetation water, the developed protocol allowed the chromatographic profile of the analytes extracted from this matrix to be evaluated, leading to satisfactory results in terms of quantitative yields. Samples of these extracts are also subjected to biological tests in order to evaluate their antioxidant and enzyme inhibition activities.

1. Introduction

The production of olive oil involves many fields of research. Many studies are focused on the enhancement and evaluation of the products and by-products of this food chain [1]. Regular consumption of olive oil in a balanced diet has been recognized as a relevant factor for a healthy life [2]. Olive oil production has experienced strong growth in recent decades as a source of antioxidants and essential fatty acids [3]. Because of this increase in production, the oil industry generates large quantities of waste from olives (wood, branches, leaves) and by-products (olive pomace, wastewater, and olive kernels), producing environmental pollution [3].

These waste products can take three different routes: be burned or used as fertilizers or discharged into the sea/lakes/rivers. This last procedure, above all, is causing a lot of damage to the environment, both for the organic component contained in it and for phytotoxicity. On the other hand, it is very expensive to dispose of these products in large quantities [4]. Many nations are working to ensure a better future by trying to reach the zero-waste target. For example, the European Union in 2020 promoted the 'circular economy action plan' with the aim of promoting sustainable consumption while minimising waste [5]. The circular economy aims to reduce the amount of waste generated by the agri-food system, enhancing and re-evaluating agricultural and food by-products (waste and non-waste), implementing recycling and moving

* Corresponding author at: Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy.
E-mail address: marcello.locatelli@unich.it (M. Locatelli).

https://doi.org/10.1016/j.jcoa.2024.100161
Received 14 June 2024; Received in revised form 17 July 2024; Accepted 18 July 2024
Available online 22 July 2024

to more sustainable production and consumption models [3].

The first step that causes waste production during the extraction of olive oil is the pruning of trees, from which large quantities of solid waste (branches, leaves, other) are generated [6]. The harvest determines the yield. The next phase is linked to handling, storage and transport process [6]. Once arrived at the mill, the olives must undergo a further cleaning phase to remove any leaves, woody parts, stones, damaged olives, dust and dirt. This phase can take place mechanically and with the use of water [7]. The next steps include grinding, blending, malaxing and separation in a centrifuge. There are two types of separation: two-phases and three-phases. From the first one, you obtain crushed olives and olive pomace (first and second design in the right part of Fig. 1); the three-phases needs water, so at the end of the process there will be 3 fractions: oil, olive pomace and waste water, as shown in Fig. 1 [8]. At the end of the process, without doubt, there will be many products of waste, for examples waste water is made by water and organic compounds, as polyphenols, tannins, lipids, sugars [9]. To enhance waste products there is a need to characterize them from a chemical-physical point of view [8]. The most important analytical parameters are physico-chemical ones, for examples physical states of matters, polyphenols and organic content. On the contrary, the content of bioactive molecules is strongly influenced by agronomic factors, climatic conditions, and variety of olives [10].

Olive pomace is the main solid by-product of olive oil extraction and represents about 35-40% of the total weight of olive processed, how previously reported, composition could have some changes [11]. Two-phases method produces semi-solid pomace more humid than that produced by the three-phase system [12]. Circular economy approaches aim to reduce the amount of waste generated within the agri-food system by enhancing and re-evaluating waste products. It is considered an alternative way of reconciling economic growth with the use of natural resources by developing innovative sustainable economic systems. These waste products contain many active compounds, the extraction of which would convert them into cheap products useful in various industrial sectors [3]. Obviously, for the extraction of these products comes into play the green chemistry (GC), whose principles require the minimization of samples, solvents and wasted energy with a consequent reduction of the final waste [13].

The enhancement of these bioactive compounds aligns perfectly with the founding principles of circular bioeconomy and with GC. The effects of these active compounds vary (antioxidant, anti-inflammatory, antibacterial), therefore the pharmaceutical industry has taken an interest both for the beneficial properties and for the enhancement of waste products.

In 2011, a study was conducted on the health benefits of polyphenols in balancing blood cholesterol levels and maintaining normal blood pressure [14]. A study on intestinal diseases is interesting, in which the anti-inflammatory properties of an aqueous extract of olive pomace in human intestinal cells have been studied. It has been noted that the inflammatory cytokine IL-8 has been reduced, confirming the inflammatory activity also on intestinal diseases [15]. Innovative approaches for the valorization of extracted polyphenols is crucial to enhance their utilization and commercial potential. These polyphenols can be utilized in the development of functional food products, dietary supplements, natural antioxidants, and nutraceutical formulations.

With the aim of extracting active compounds from these waste products, we developed a method based on a green extracting method: ultrasound assisted extraction (UAE). As previously reported, these active compounds can be used in many application areas, from cosmetic to medical field. In addition, this work could be a possible approach of matching GC with bioeconomy, reducing waste, which is a common goal, and reusing waste material for other companies. Moreover, Green Sample Preparation (GSP) require specifically maximizing sample productivity, and with this approach GSP principles are followed.

2. Materials and methods

2.1. Chemicals, solvents and devices

Gallic acid, catechin, chlorogenic acid, *p*–OH benzoic acid, vanillic acid, epicatechin, syringic acid, 3–OH benzoic acid, 3–OH–4–MeO benzaldehyde, *p*–coumaric acid, rutin, sinapinic acid, *t*–ferulic acid, naringin, 2,3–diMeO benzoic acid, benzoic acid, *o*–coumaric acid, quercetin, harpagoside, *t*–cinnamic acid, naringenin, and carvacrol were purchased from Sigma Aldrich (Milan, Italy). Methanol and acetonitrile (AcN) (HPLC–grade) and acetic acid (99%) were obtained from Carlo Erba Reagenti (Milan, Italy). Milli–Q water was obtained using a Millipore Milli–Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

The freeze dryer used is a system Epsilon LCG LYO CHAMBER GUARO acquired by Martin Christ (German). Sonicator was purchased from Parmer Instrument (England) with 100 W power. During the concentration's phase of samples, SpeedVac Concentrator SC110A Savant from Eco Vide (Italy) was used.

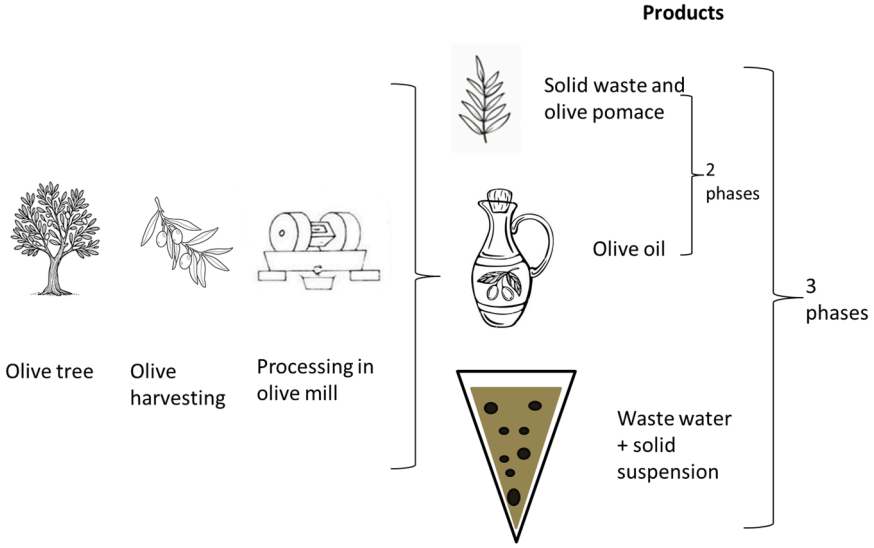

Fig. 1. Representation of the process leading to olive oil, olive pomace and waste water.

2.2. HPLC conditions

HPLC analyses were performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector (PDA), and Empower *v.2* Software (Waters Spa, Milford, MA, USA) was used for acquisition of data. A C_{18} reversed–phase packing column (Prodigy ODS (3), 4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA, USA) was used for the separation and the column was thermostated at 30 ± 1°C using a Jetstream2 Plus column oven. The UV/Vis acquisition wavelength was set in the range of 200–500 nm. The quantitative analyses were achieved at maximum wavelength for each compound. The injection volume was 20 µL. The mobile phase was directly *on–line* degassed by using Biotech DEGASi, mod. Compact (LabService, Anzola dell'Emilia, Italy). Gradient elution was performed using the mobile phase water-acetonitrile (93:7, *v:v*, 3% acetic acid) as reported in ***Supplementary material S.1***. [16,17].

2.3. Sampling and sample preparation

Sampling was carried out in random mode on the same day as the entire production of the oil lot. The samples were taken from appropriate tanks containing the waste material and packaged in 50 mL plastic tubes. They were stored until the freeze-drying procedure at +4°C and in the dark. Type of product, origin, processing and production are shown in Table 1.

To maximize the extraction efficiency and polyphenol recovery from olive oil mill waste, design of experiments methodology was employed [18,19]. A factorial design with three factors at three levels was constructed to investigate the effects of key extraction parameters, including: percentage composition of extracting phase (X1), extraction time (X2), and volume of the extraction phase (X3). The experimental design matrix was generated based on the selected factors, and the extraction experiments were carried out accordingly. The resulting extracts were analyzed using HPLC to determine the polyphenolic composition and yield. To further refine the extraction conditions and determine the optimum values for the extraction parameters, Response Surface Methodology (RSM) was employed. RSM involves the creation of a mathematical model that predicts the response (polyphenol yield) based on the experimental factors and their levels. The use of contour plots and response surface plots, the RSM approach allowed for a comprehensive visualization of the relationship between the extraction parameters and polyphenol yield.

3. Results and discussion

Firstly, it was noticed that water content in each processing was different. After freeze-drying, pomace obtained from two-phases processing had lost 70% by weight; pomace from two-phases and half 50% and 45% for three-phases pomace.

Solid liquid extraction and ultrasound-assisted extraction (UAE) were initially tested, to evaluate the best extraction method. These tests showed that the solid-liquid extraction assisted by ultrasound showed a yield of polyphenolic compounds much higher than the corresponding extraction with magnetic stirring. Furthermore, by keeping the temperature stable at 25°C, the degradation of the analytes of our interest is minimized. In fact, extractions carried out above 40°C led to the degradation of the latter (data not showed). Thus, with the use of UAE and ambient temperature energy and solvent waste are minimized, following the principles of GC [13].

Table 1
Product, origin, processing and production.

Product	Origin	Processing	Production
Olive pomace	42°18′15.7″N 14°10′50.02″E	Two-phases	On continuus
Olive pomace Wastewater	42°19′20.17″N 14°02′43.98″E	Two-phases and half	On continuus
Olive pomace Wastewater	41°37′17″N 14°52′34″E	Three-phases	On continuus

Thus, an exact volume of extract solution containing water (H_2O) and ethanol (EtOH) was added to 1g of dried pomace. The mixture was then subjected to UAE in a dark room. The extract was, then, centrifuged for 10 min. at 14000 rpm. A fixed volume (1.5 mL) of supernatant has been dried through SpeedVac. The choice of EtOH and water is also based on the characteristics of solvents, as they are considered 'green' [13].

The wastewater was treated in order to be separated as much as possible from the suspended solid phase and to evaluate its chromatographic profile for the presence of polyphenolic bioactive compounds. Water suspension particles are decanted as far as possible to separate the solid part from the aqueous part. After a first separation by decantation, the water is centrifuged at 5000 rpm for 10 min. The supernatant liquid is then separated from the suspension solid. The solid will be treated with the extraction procedure described above. The aqueous supernatant is further filtered to remove further solid residues. The residual liquid is then freeze-dried and maintained in dark room at -20°C.

About pomace olive and suspended solid phase, they were suspended in 100 µL of mobile phases, vortexed, subjected to ultrasound for 3 min. and vortexed again. Finally, after a centrifuge of 10000 rpm for 10 min., the supernatant was injected into HPLC. Using these abovementioned preliminary tests, the experimental ranges of the individual variables to be studied were selected. Each variable has three study levels indicated with -1, 0 and +1 corresponding to the lower, intermediate and upper levels respectively, as reported in ***Supplementary material S.2***.

After carrying out the experimental tests inherent in the design, the selected variables were optimized with the quadratic model by the formation of a Response Surface. Through the latter procedure, it was possible to determine the best experimental conditions to carry out the extraction process and verify the actual influence of the selected variables. In order to optimize, the one from the two and a half phases processing has been selected; this allows us to have a matrix of intermediate composition compared to the different types of processing of olive oil.

The influence of temperature on the extraction yield has been evaluated; various tests carried out at different temperatures have shown a decrease in yield as the temperature increases. Tests carried out above 30°C have led to a sharp decrease in the concentration of our analytes in the extracts, while above 40°C the analytes are almost totally absent [16,17,20]. Therefore, it was decided to proceed with the extractions while maintaining the temperature at 25°C. Further tests were carried out to assess the influence of the type of agitation adopted during the extraction. In particular, the magnetic agitation was compared with the use of ultrasound: maintaining constant other experimental conditions, the ultrasound-assisted extraction proved to be much higher in terms of yield.

Subsequent tests were aimed at investigating the composition of the mining phase. From a solution containing only ethanol, the low extraction capacity of this extraction phase was found. Subsequently, tests were carried out with an extracting mixture consisting of H_2O and EtOH with a percentage ratio of 70:30 (*v:v*) and 60:40 (*v:v*). Both these phases have led to the formation of colloidal suspensions in extracts, which are not compatible with chromatographic analysis. They also showed very low extraction capacity. These results have oriented the choice of the experimental interval to be studied concerning the composition of the extracting mixture towards a 50:50 (*v:v*) composition of H_2O:EtOH.

At the end time of extraction was evaluated. As the extraction time increases, initially there is an increase in yield that, reached the maximum peak, tends to decrease progressively until it cancels over the two hours of extraction. These results have directed the choice of the interval of study of this variable in a range from 30 min. to 90 min. of

extraction.

The experimental conditions inherent to the points of the experimental design are as follows, where -1 represents the lower level, 0 the intermediate and +1 the upper level, as shown in ***Supplementary material S.2***. In Table 2, there are results obtained from these experiments expressed in μg/g, for each bioactive compound resulting from the experiments and the total amount of polyphenols found.

By analysing the experimental results obtained, it was concluded that he operational process developed allows us to identify and quantify 9 analytes of our interest. Certain experimental conditions allow us to have a good extraction yield of the total amount of polyphenolic compounds in the extracts, with a maximum of 78.38 μg/g. By examining the yields of the individual families of compounds, there are different quantitative trends depending on the test conditions.

The qualitative analysis of extracts allowed us to identify 9 polyphenolic compounds within the sensitivity range of the HPLC analysis method. The analytes identified are as follows: gallic acid, *p*-OH-benzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, sinapinic acid, *o*-coumaric acid, hydrated catechin and 3-OH-4MeO-benzaldehyde.

Because of these experiments, replicate tests have been carried out outside the selected experimental range, in order to investigate the response outside the set conditions. Specifically, replicate extractions were carried out under the following conditions: 50:50 water: ethanol of the extracting phase, 15 mL of the extracting phase, and 20 min. of extraction. Under these conditions, the yield was lower than the maximum quantity obtained under the conditions of the experimental design, specifically the total quantity of polyphenols determined is 33.52 μg/g in mean value of the replicates.

The obtained data from the DoE experiments were subjected to statistical analysis using analysis of variance (ANOVA). This analysis allowed for the assessment of the main effects and interaction effects of the extraction parameters on polyphenol yield. Two quadratic models were formulated, the first for the optimization of the extraction of the total amount of polyphenolic compounds, the second for the optimization of the extraction of polyphenolic compounds derived from benzoic acid. The statistical significance of the factors and their interactions was evaluated to identify the most influential parameters in the extraction process. Based on the statistical analysis, a mathematical model was developed to predict the polyphenol yield as a function of the extraction process variables. The model provided valuable insights into the relationship between the extraction parameters and the recovery of polyphenols. It also allowed for the optimization of the extraction conditions by predicting the optimal parameter values that would maximize polyphenol recovery. The tables in ***Supplementary material S.3*** show the statistical analysis related to the developed methods.

In Fig. 2 were reported the graph of agreement between the predicted and experimental data (a) related to the extraction conditions of the total amount of polyphenols and the model response surface (b). Similarly, in the Fig. 3 were also reported the graph of agreement between the expected and experimental data (a) related to the optimization of the extraction of polyphenolic compounds derived from benzoic acid and the model response surface (b).

The maximum quantitative yield of the total sum of the polyphenolic compounds is obtained by extraction with an extraction phase volume of 15 mL for an extraction time of 30 min. In this model, we have a cumulative response, in which we find the response of both the most polar and the most apolar compounds. The most abundant compounds corresponding to benzoic acid derivatives give the major contribution of the model. Analysing the statistical parameters the model presents a good R^2 (0.839) with a good agreement between the expected data and those observed. In terms of the significance of the parameters, it is observed that the first variable (X1) is not significant, while the second variable (X2) and the third variable (X3) have a relevant significance on the response. This figure is justified by the fact that the experimental range of the composition of the extractive phase was rather limited. Therefore, a significant variation of the response according to the composition of the extractor is not appreciable. Studying the curvature of the response surface, it is evident that the second variable has a more marked trend than the third variable; this shows that the extraction time is the most significant variable for the optimization. Extraction under these test conditions allows a total quantity of polyphenols of 77.01 μg/g to be extracted with a standard error of 1.37 μg/g, which is very close to the

Table 2
Results obtained following the DoE experimental design.

Test	Gallic acid	*p*-OH-benzoic acid	Vanillic acid	Syringic acid	*p*-coumaric acid	Sinapinic acid	*o*-coumaric acid	Catechin	3-OH-4-MeO-benzaldehyde	Total amount of polyphenols (μg/g)
1	0.92	2.01	30.42	0.29	21.83	0.75	0.15	0.56	3.03	59.95
2	1.30	2.08	32.99	0.39	23.62	1.28	0.4	0.54	2.76	65.35
3	1.41	1.76	29.51	0.27	20.68	1.12	0.20	0.53	2.88	58.36
4	6.99	0.71	6.91	0.74	4.47	0.75	0.12	0.31	3.18	24.19
5	10.65	0.88	8.60	1.07	6.49	1.10	0.25	0.45	1.31	30.81
6	1.56	1.61	26.68	0.26	20.85	1.10	0.36	0.47	2.39	55.29
7	2.01	0.51	6.36	0.79	4.38	0.66	0.18	0.55	0.84	16.28
8	3.54	0.61	6.02	0.90	5.27	0.81	0.23	0.47	1.71	19.56
9	5.32	0.84	7.53	1.26	6.08	0.85	0.28	1.67	5.72	29.55
10	1.28	1.90	34.09	0.33	23.31	0.82	0.15	0.60	4.37	66.84
11	1.36	2.14	36.98	0.40	28.75	1.46	0.41	0.60	2.97	75.08
12	1.46	2.14	36.89	0.40	30.51	1.60	0.25	0.71	4.43	78.38
13	9.08	0.64	7.72	0.87	4.65	0.89	0.13	0.33	0.87	25.18
14	10.73	0.70	7.45	0.98	8.99	1.08	0.24	0.50	1.57	32.24
15	10.08	0.68	7.56	1.02	5.85	0.92	0.27	0.83	1.14	28.34
16	8.19	0.86	7.70	0.63	10.07	0.62	0.22	0.89	2.22	31.39
17	1.35	2.28	32.11	0.34	30.62	1.55	0.44	0.66	3.25	72.59
18	1.98	0.68	7.39	1.21	5.06	0.81	0.18	0.38	0.92	18.61
19	2.67	0.56	5.95	0.72	5.24	0.80	0.23	0.97	3.67	20.81
20	5.42	0.66	6.20	0.92	5.18	0.72	0.27	1.39	3.72	24.48
21	1.28	2.07	34.32	0.39	25.69	0.89	0.25	0.62	3.72	69.23
22	1.65	2.03	30.78	0.28	18.32	1.17	0.33	0.51	3.98	59.05
23	1.25	2.34	27.41	0.29	23.86	1.27	0.22	0.66	3.72	61.03
24	9.39	0.60	7.61	0.58	5.49	0.94	0.14	0.29	1.29	26.32
25	8.32	0.65	6.50	0.78	6.15	0.96	0.23	0.42	1.27	25.27
26	0.78	2.22	31.41	0.37	25	1.35	0.38	0.51	2.55	64.57
27	2.46	0.51	6.07	0.68	5.5	0.70	0.16	0.59	0.78	17.44
28	4.35	0.64	7.18	1.05	5.61	0.92	0.25	1.44	3.40	24.83
29	5.78	0.71	6.79	0.93	6.34	0.83	0.27	1.58	4.25	27.66

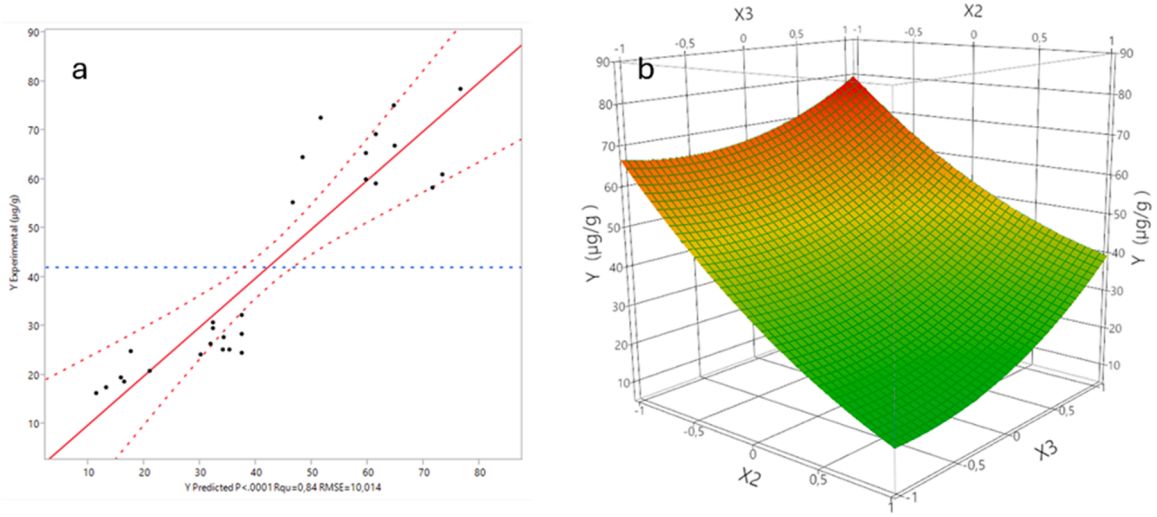

Fig. 2. Plot of experimental vs. predicted values in regression model (a) related to the extraction conditions of the total amount of polyphenols and relative response surface plot (b) for yield extraction estimated by plotting extraction time (X2) versus volume of the extraction phase (X3).

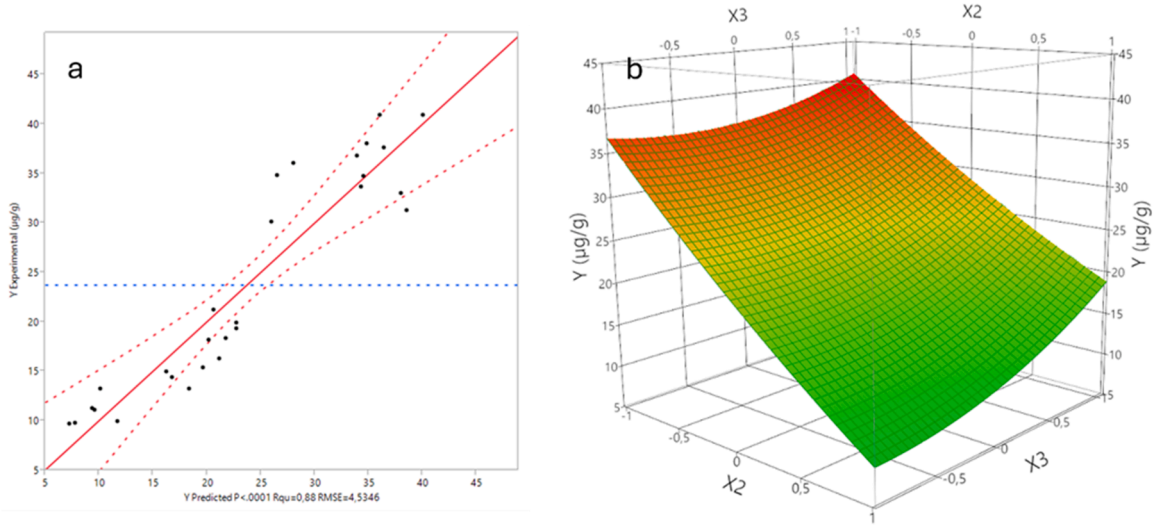

Fig. 3. Plot of experimental vs. predicted values in regression model (a) related to the extraction conditions of polyphenolic compounds derived from benzoic acid and relative response surface plot (b) for yield extraction estimated by plotting extraction time (X2) versus volume of the extraction phase (X3).

one calculated with the model.

The second optimization model is related to the extraction conditions of polyphenolic compounds derived from benzoic acid, a family of compounds with the highest number of analytes present in extracts. Specifically, the analytes in question are gallic acid, *p*-OH-benzoic acid, vanillic acid, and syringic acid. The following tables show the statistical analysis linked to the developed method. In ***Supplementary material S.3*** there are the descriptive parameters of the models. The maximum yield of polyphenolic compounds derived from benzoic acid is obtained by extraction with an extraction phase volume of 15 mL for an extraction time of 30 min.

The model in question resulted in optimized conditions coinciding with the model relating to the total amount of polyphenols. Analysing the statistical parameters the model presents a good R^2 (0.877) with a satisfactory agreement between the expected data and the experimental ones. According to the first model, in terms of the significance of the parameters, it is observed that the first variable (X1) is not significant, while the second variable (X2) and the third variable (X3) have a significant significance on the response. By analysing the shape of the Response Surface, it was possible to deduce that the second variable has a more marked trend than the third variable. In addition, in this case the extraction time represents the most significant variable. Under these conditions of extraction, a yield of the compounds of interest of 40.22 µg/g in mean value of the replicates is obtained with a standard error corresponding to 0.67 µg/g, also in this case very close to the calculated value.

In Table 3 were reported the final results from the quantitative analysis of the different sample-types (n=3) carried out under the optimized conditions, while in ***Supplementary material S.4*** were reported the chromatograms of the analyses.

The optimised extraction procedure was then applied to the other sampled husks and to the suspended solids of the vegetation water. Specifically, the method has been applied to the husks produced by the two-phase and three-phase process, and in addition to the solids of the vegetable water taken from the two-phase and three-phase mills. The analytes identified in the extracts of the two-phase pomace are gallic acid, hydrated catechin, *p*-OH-benzoic acid, vanillic acid, syringic acid, 3-OH-benzoic acid, 3-OH-4-MeO-benzaldehyde acid, *p*-coumaric acid, rutin, sinapinic acid, naringin, *o*-coumaric acid.

The analytes identified in the extracts of the three-phase pomace are

Table 3
Analytes identified in the extracts of oil pomace, suspension solid from wastewater and vegetation water samples, expressed in μg/g.

Product	Type of pomace	Gallic acid	Catechin	p-OH benzoic acid	Vanillic acid	Epicatechin	Syringic acid	3-OH benzoic acid	3-OH-4-MeO-benzaldeide	p-cumaric acid	Rutin	Sinapinic acid	Naringin	o-cumaric acid	t-cinnamic acid	Benzoic acid	Tot (μg/g)
Olive pomace	**2 phases**	2.75 (±0.18)	85.62 (±4.25)	9.78 (±0.38)	373.03 (±14.40)		22.29 (±0.88)	9.48 (±0.64)	12.36 (±0.48)	879.36 (±33.74)	3.89 (±0.45)	12.14 (±0.17)	0.84 (±0.05)	1.21 (±0.01)			1412.75 (±52.95)
	2 and half phases	1.23 (±0.22)	0.63 (±0.08)	2.45 (±0.31)	35.96 (±0.93)		0.58 (±0.18)		3.09 (±1.33)	31.20 (±0.68)		1.60 (±0.01)		0.26 (±0.02)			77.01 (±1.37)
	3 phases	1.50 (±0.03)	6.03 (±0.04)	3.31 (±0.01)	34.46 (±0.48)	1.94 (±0.01)	0.71 (±0.03)	5.81 (±0.06)		99.81 (±1.18)		1.64 (±0.02)		0.28 (±0.01)	0.38 (±0.01)		155.86 (±1.82)
Suspension solid from wastewater	**2 and half phases**	13.62 (±0.61)	33.52 (±0.66)	16.39 (±0.82)	230.28 (±11.89)	2.03 (±0.22)	3.48 (±0.16)	29.26 (±0.40)		16.71 (±0.12)						8.63 (±0.08)	353.92 (±14.72)
	3 phases	3.92 (±0.01)	51.48 (±1.39)		334.82 (±20.17)	52.57 (±1.01)	4.48 (±0.08)	20.41 (±0.23)		1500.95 (±82.60)							1969.14 (±105.53)
Vegetation water samples	**2 and half phases**	53.73 (±1.72)	458.32 (±7.75)	44.40 (±0.23)	766.76 (±2.45)						195.29 (±10.77)					40.50 (±5.22)	1559.00 (±15.15)
	3 phases		274.85 (±1.39)				117.47 (±1.44)			3619.32 (±26.58)		50.36 (±5.77)					4062.01 (±23.24)

gallic acid, hydrated catechin, *p*-OH-benzoic acid, vanillic acid, epicatechin, syringic acid, 3-OH-benzoic acid, *p*-coumaric acid, sinapinic acid, *o*-coumaric acid, *t*-cinnamic acid. This extraction procedure allows us to extract 12 polyphenolic analytes from the two-phase pomace, while for the three-phase pomace 11 polyphenolic analyte extracts have been identified. The new analytes have been identified in these extracts, as opposed to extracts obtained from two-and-a-half-phase olive residue, which fall within the sensitivity range of the method of analysis. The optimised extraction conditions resulted in a good yield of the total amount of polyphenolic compounds, 1412.75 (±52.95) μg/g for two-phase olive residue and 155.86 (±1.82) μg/g for three-phase olive residue respectively.

The same model was subsequently applied to the suspension solids of the vegetation waters sampled in the two-and-a-half and three-phase mills. The analytes identified in the extracts from the suspension solid of the crusher with two and a half phases are gallic acid, hydrated catechin, *p*-OH-benzoic acid, vanillic acid, epicatechin, syringic acid, 3-OH-4-MeO-benzaldehyde, *p*-coumaric acid, benzoic acid. The analytes identified in the extracts from the suspension solid of the three-phase crusher are gallic acid, hydrated catechin, vanillic acid, epicatechin, syringic acid, 3-OH-4-MeO-benzaldehyde, *p*-coumaric acid.

This extraction procedure allows us to extract 9 polyphenolic analytes from the two-and-a-half-phase solid water, while 7 polyphenolic analytes have been identified in extracts from the three-phase solid water. New analytes have been identified in these extracts, as opposed to extracts obtained from two-and-a-half-phase olive residue, which fall within the sensitivity range of the method of analysis.

The optimised extraction conditions resulted in a good yield of the total amount of polyphenolic compounds, respectively 353.92 (±14.72) μg/g for the first solid and 1969.14 (±105.53) μg/g for the second solid. The same method has been applied to two-and-a-half and three-stage wastewater. The analytes identified in the two and a half phase vegetation water samples are gallic acid, hydrated catechin, *p*-OH-benzoic acid, vanillic acid, rutin, benzoic acid. The analytes identified in the three-phase vegetation water samples are catechin hydrate, syringic acid, *p*-coumaric acid, and sinapinic acid. All the possible application of these analytes are shown in Table 4.

Table 4
Possible application.

Analyte	Properties
Gallic acid	antioxidant, anti-inflammatory in gastrointestinal, neuropsychological, metabolic, and cardiovascular disorders
Catechin	Antimicrobial, antiviral, antiallergic. It increase absorption of healthy foods and cosmetics into body and skin.
p-OH benzoic acid	antimicrobial, antialgal, hypoglycaemic, anti-inflammatory, preservative in many cosmetic and pharmaceuticals products and food
Vanillic acid	preservative, flavoring agent, and a food additive
Epicatechin	Antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, cardioprotective
Syringic acid	Antioxidant, anti-inflammatory, antiendotoxic, neuro, cardio and hepatoprotective
3-OH- benzoic acid	Antifungal, food additives,
3-OH-4-MeO-benzaldehyde	Can be used coupled with other bioactives in order to improve the total biological activities
p-coumaric acid	Antioxidant, antitumor, antibacterial, antifungal
Rutin	Prevention of neuroinflammatiom, anticonvulsant, suppressing activity of cytokines, antidepressant effects
Sinapinic acid	Antioxidant, anti-inflammatory, anti-cancer, reno-, neuro- and cardio-protective, antibacterial, anxiolytic
Naringin	Antiproliferation for cancer, increase levels of glucose in blood, hepatoprotective, protection against nickel toxicity, protective against some neurological disease, increasing of osteogenic differentiation.
o-coumaric acid	Anticarcinogenic activities, antioxidant,
t-cinnamic acid	Antioxidant, anti-inflammatory, anticancer activities.
Benzoic acid	Antibacterial and antifungal activity, improving gut function.

With the optimized method there is a cumulative response, in which we find the response of both the most polar and the most apolar compounds, in which major contribute is given by benzoic acid's derivates, as gallic acid, *p*-OH-benzoic acid, vanillic acid and syringic acid. Analysing the statistical parameters the model presents a good R^2, with a good agreement between the expected data and those observed. In terms of the significance of the parameters, it is observed that the first variable (X1) is not significant, while the second variable (X2) and the third variable (X3) have a relevant significance on the response. This figure is justified by the fact that the experimental range of the composition of the extractive phase was rather limited, therefore a significant variation of the response according to the composition of the extractor is not appreciable. Studying the curvature of the Response Surface, it is evident that the second variable has a more marked trend than the third variable; this shows that the extraction time is the most significant variable for the optimization for both, polyphenols and benzoic acid's derivates. About polyphenols, extraction with optimized method obtained 77.01 μg/g with an error of 1.37 μg/g, value very close to that calculated with the model. Instead, in the optimized conditions, for benzoic acid derivates a yield equal to 40.22 μg/g was obtained with an error of 0.67 μg/g, also in this case value too close to the model.

We tested on the reported procedure, the Analytical GREEnness Metric (AGREE), Analytical greenness metric for sample preparation (AGREEprep), and Blue Applicability Grade Index (BAGI), that indicates the green profile and the practicability of the method used, obtaining a good score, as shown in Fig. 4 [21].

About greenness of the method, the possibility to reuse a waste product, firstly, matches with GC principles, that are based on minimizing waste [13]. The extraction method, using UAE, is less impacting on the environment in terms of both energy and solvent quantity. The large number of analytes allows quantifying more than 15 in a single run with different chemical characteristics, which is why it was decided to use a gradient method, in this way also derivatization is avoided. The choice of solvent extraction as ethanol and water was based on GC principles, how previously reported, and they result are safe for personnel.

After this analytical characterization, we considered essential to implement the data with a biological characterization, testing pomace, suspension solid and wastewater. The data presented in the Table 5 provides a comprehensive overview of the antioxidant capacities and enzyme inhibition potentials of various samples derived from olive processing, specifically focusing on different phases of olive pomace, water suspension solids, and olive oil wastewater. The results are indicative of how the method of processing and the specific phase of extraction can markedly influence the bioactive properties of the resulting materials. In terms of antioxidant activities assessed by DPPH, FRAP, and phosphomolybdenum assays, it is notable that the 'Olive pomace (two and half phases)' sample displayed the highest antioxidant activity in both the DPPH and FRAP assays with values of 53.29 mg ascorbic acid equivalent (AAE)/g and 56.82 mg gallic acid equivalent (GAE)/g, respectively. This suggests that the extraction method used in this phase effectively concentrates antioxidant compounds, potentially making it a valuable source of natural antioxidants for food and pharmaceutical applications. The high score in the phosphomolybdenum assay (18.42 mg GAE/g) further underscores its robust capacity to reduce oxidative stress. In contrast, 'Olive oil wastewater (three phases)' exhibited the lowest antioxidant activity in the DPPH assay, which might reflect a lesser concentration of active reducing agents or a degradation of phenolic compounds during this particular phase of processing.

The enzyme inhibition assays reveal that 'Olive pomace (two and half phases)' again shows superior performance, particularly in inhibiting tyrosinase with an impressive value of 172.19 mg Kojic acid equivalent (KAE)/g. Such a high inhibition rate is significant as it highlights the potential of using this byproduct in cosmetic formulations to prevent hyperpigmentation. Similarly, its acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition values are among the highest recorded in the table, suggesting its potential use in the management of neurodegenerative diseases, such as Alzheimer's disease, where these enzymes are therapeutic targets.

'Water suspension solid (two and half phases)' exhibits an interesting profile with the highest inhibition of AChE (6.32 mg GALAE/g), which is considerably higher than other samples. This could be particularly relevant in the development of treatments for neurodegenerative conditions. However, its tyrosinase inhibition is relatively lower, indicating that its enzyme inhibition profile may be more selective compared to other samples.

The variability observed across different samples and phases indicates that the biochemical properties of byproducts from olive oil production are significantly influenced by the processing techniques and conditions. This suggests that optimizing these conditions could tailor the extracts for specific industrial applications, maximizing the utility of olive byproducts and contributing to more sustainable production practices. Overall, these results provide a valuable insight into the potential applications of olive processing byproducts, displaying their capabilities as natural sources of antioxidants and enzyme inhibitors. Future research could further optimize processing methods to enhance these properties, expanding the economic and environmental benefits of olive agriculture.

Conclusion

By assessing its effectiveness and its compatibility with the objectives previously set, the protocol described provided a new and alternative extraction methodology compared to the traditional methods used, providing interesting insights into possible future studies. The optimised

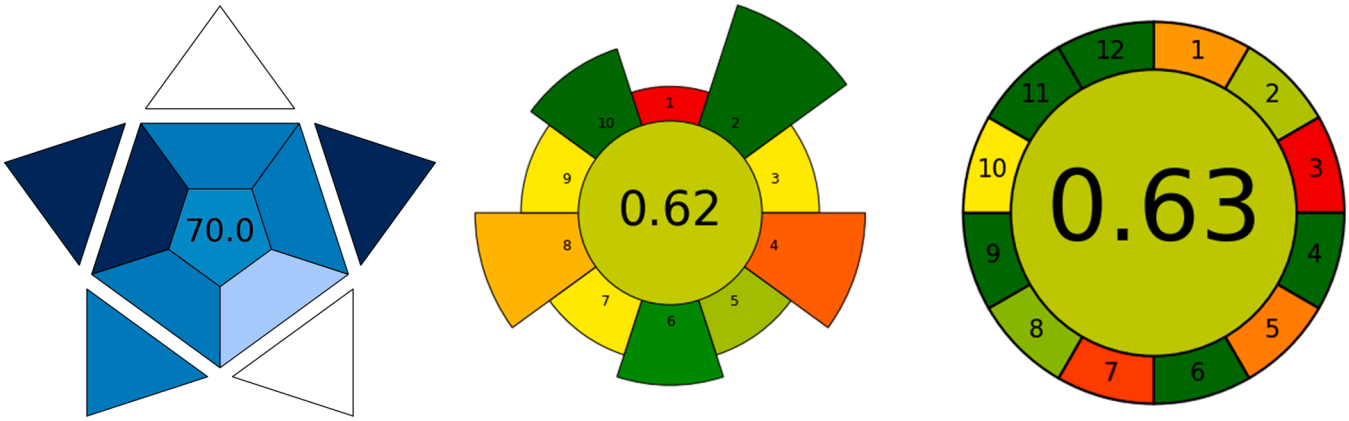

Fig. 4. BAGI (left), AGREEprep (centre), and AGREE (right) pictograms and scores for the proposed procedure.

Table 5
Biological studies (antioxidant activities and enzyme inhibition potential) of the tested samples.

Samples	Antioxidant activities			Enzyme inhibition assays		
	DPPH (mg AAE/g)	FRAP (mg GAE/g)	Phosphomolybdenum (mg GAE/g)	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)
Olive pomace (two phases)	39.15±0.72	48.49±1.02	9.54±0.83	2.19±0.09	1.09±0.82	87.92±0.93
Olive pomace (two and half phases)	53.29±1.05	56.82±0.84	18.42±0.91	4.16±0.72	3.92±1.10	172.19±1.19
Olive pomace (three phases)	31.26±0.93	39.03±0.72	11.43±1.03	2.93±0.91	1.99±0.18	113.41±0.83
Water suspension solid (two and half phases)	34.58±0.43	45.39±1.31	15.39±0.73	6.32±0.41	4.52±0.84	43.31±0.94
Water suspension solid (three phases)	47.62±0.91	26.49±0.73	29.83±0.51	1.92±0.86	2.06±0.49	95.42±1.02
Olive oil wastewater (two and half phases)	37.32±1.03	35.82±0.71	24.41±1.01	5.61±0.62	3.39±0.82	71.09±0.83
Olive oil wastewater (three phases)	21.43±0.28	29.72±1.62	17.82±0.71	4.43±0.85	3.87±0.39	84.59±1.04

AAE: ascorbic acid equivalent; GAE: gallic acid equivalent; GALAE: Galantamine equivalent; KAE: Kojic acid equivalent; AChE:: acetylcholinesterase, BChE: butyrylcholinesterase

extraction methodology, starting from the two-and-a-half-phase olive pomace, has proved satisfactory in terms of efficiency by evaluating the effect of parameters such as extraction time and process temperature. The application of this methodology to other types of pomace and by-products has shown excellent results in terms of extractive yield, demonstrating the validity of this procedure as suitable for other solid residues from the oil industry. With regard to the treatment in the waters of vegetation, the protocol developed has allowed to evaluate the chromatographic profile of the analytes extracted from this matrix leading to satisfactory results in terms of quantitative yields. Additionally, there is an increase in the interest of reporting safety and effect of natural product, above all if these substances are waste products, which only would create pollution and waste. For all the reasons above mentioned, the method developed can be an excellent starting point for study, as it affects various areas of study, from the field of bioeconomy to GC, through the GSP. Thanks to minimizing waste and maximizing sample productivity, many approaches could start from this research, hoping for an increasingly 'greener' chemistry.

CRediT authorship contribution statement

Miryam Perrucci: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Marco Dezio:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Hammad Saleem:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Fabrizio Ruggieri:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Marcello Locatelli:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization.

Declaration of competing interest

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

Funding

Marcello Locatelli acknowledges financial support funded by the European Union – NextGenerationEU, under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 - M4C2, Investment 1.5 – Call for tender No. 3277 of 30.12.2021, Italian Ministry of University, Award Number: ECS00000041, Project Title: "Innovation, digitalization and sustainability for the diffused economy in Central Italy", Concession Degree No. 1057 of 23.06.2022 adopted by the Italian Ministry of University. CUP: D73C22000840006.

Data availability

Data will be made available on request.

Acknowledgements

Authors would like to thank all the respective Universities for the support provided in the literature survey.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2024.100161.

References

[1] P. Otero, P. Garcia-Oliveira, M. Carpena, M. Barral-Martinez, F. Chamorro, J. Echave, P. Garcia-Perez, H. Cao, J. Xiao, J. Simal-Gandara, M.A. Prieto, Applications of by-products from the olive oil processing: Revalorization strategies based on target molecules and green extraction technologies, Trends Food Sci. Technol. 116 (2021) 1084–1104, https://doi.org/10.1016/j.tifs.2021.09.007.
[2] M. Orive, M. Cebrián, J. Amayra, J. Zufía, C. Bald, Integrated biorefinery process for olive pomace valorisation, Biomass Bioenerg. 149 (2021) 106079, https://doi.org/10.1016/j.biombioe.2021.106079.
[3] M. Donner, I. Radić, Innovative circular business models in the olive oil sector for sustainable mediterranean agrifood systems, Sustainability 13 (5) (2021) 1–21, https://doi.org/10.3390/su13052588.
[4] P. Gullon, B. Gullon, G. Astray, M. Carpena, M. Fraga-Corral, M.A. Prieto, J. Simal-Gandara, Valorization of by-products from olive oil industry and added-value applications for innovative functional foods, Food Res. Int. 137 (2020) 1–17, https://doi.org/10.1016/j.foodres.2020.109683.
[5] M. Donner, Y. Erraach, F. López-I-Gelats, J. Manuel-i-Martin, T. Yatribi, I. Radić, F. El Hadad-Gauthier, Circular bioeconomy for olive oil waste and by-product valorisation: Actors' strategies and conditions in the Mediterranean area, J. Environ. Manage. 321 (2022) 1–12, https://doi.org/10.1016/j.jenvman.2022.115836.
[6] B. Gullón, P. Gullón, G. Eibes, C. Cara, A. De Torres, J.C. López-Linares, E. Ruiz, E. Castro, Valorisation of olive agro-industrial by-products as a source of bioactive compounds, Sci. Total Environ. 645 (2018) 533–542, https://doi.org/10.1016/j.scitotenv.2018.07.155.
[7] J. Madureira, F.M. Margaça, C. Santos-Buelga, I.C. Ferreira, S.C. Verde, L. Barros, Applications of bioactive compounds extracted from olive industry wastes: A review, Compr. Rev. Food Sci. Food Saf. 21 (1) (2022) 453–476, https://doi.org/10.1111/1541-4337.12861.
[8] M.J. Negro, P. Manzanares, E. Ruiz, E. Castro, M. Ballesteros, The biorefinery concept for the industrial valorization of residues from olive oil industry, in Olive Mill Waste - Recent Advances for Sustainable Management, Elsevier. Chapter 3 (2017) 57–78, https://doi.org/10.1016/B978-0-12-805314-0.00003-0.
[9] F. Sciubba, L. Chronopoulou, D. Pizzichini, V. Lionetti, C. Fontana, R. Aromolo, S. Socciarelli, L. Gambelli, B. Bartolacci, E. Finotti, A. Benedetti, A. Miccheli, U. Neri, C. Palocci, D. Bellincampi, Olive mill wastes: A source of bioactive molecules for plant growth and protection against pathogens, Biol 9 (12) (2020) 1–20, https://doi.org/10.3390/biology9120450.
[10] G. Difonzo, M. Troilo, G. Squeo, A. Pasqualone, F. Caponio, Functional compounds from olive pomace to obtain high-added value foods–a review, J. Sci. Food Agric. 101 (1) (2021) 15–26, https://doi.org/10.1002/jsfa.10478.

[11] J.M. Romero-García, L. Niño, C. Martínez-Patiño, C. Álvarez, E. Castro, M.J. Negro, Biorefinery based on olive biomass State of the art and future trends, Bioresour. Technol. 159 (2014) 421–432, https://doi.org/10.1016/j.biortech.2014.03.062.
[12] M. Matos, M.F. Barreiro, A. Gandini, Olive stone as a renewable source of biopolyols, Ind. Crops Prod. 32 (1) (2010) 7–12, https://doi.org/10.1016/j.indcrop.2010.02.010.
[13] M. Locatelli, A. Kabir, M. Perrucci, S. Ulusoy, H.I. Ulusoy, I. Ali, Green profile tools: current status and future perspectives. Advances in Sample Preparation, 6 (2023) 100068, 1-15. https://doi.org/10.1016/j.sampre.2023.100068.
[14] EFSA Panel on Dietetic Products, N. and Allergies, Scientific opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781),"anti-inflammatory properties"(ID 1882),"contributes to the upper respiratory tract health"(ID 3468),"can help to maintain a normal function of gastrointestinal tract"(3779), and "contributes to body defences against external agents"(ID 3467) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. EFSA journal 9(4) (2011) 2033, 1-25. https://doi.org/10.2903/j.efsa.2011.2033.
[15] M. Di Nunzio, G. Picone, F. Pasini, M.F. Caboni, A. Gianotti, A. Bordoni, F. Capozzi, Olive oil industry by-products. Effects of a polyphenol-rich extract on the metabolome and response to inflammation in cultured intestinal cell, Food Res. Intern. 113 (2018) 392–400, https://doi.org/10.1016/j.foodres.2018.07.025.
[16] C. Ferrante, L. Recinella, M. Locatelli, P. Guglielmi, D. Secci, L. Leporini, A. Chiavaroli, S. Leone, S. Martinotti, L. Brunetti, M. Vacca, L. Menghini, G. Orlando, Protective effects induced by microwave-assisted aqueous Harpag (ophytum extract on rat cortex synaptosomes challenged with amyloid β-peptide, Phytother. Res. 31 (2017) 1257–1264, https://doi.org/10.1002/ptr.5850.
[17] A. Mocan, M. Babota, A. Pop, I. Fizeşan, A. Diuzheva, M. Locatelli, S. Carradori, C. Campestre, L. Menghini, C.R. Sisea, M. Soković, G. Zengin, P. Ramona, A.S. Badarau, D.C. Vodnar, G. Crişan, Chemical constituents and biological activities of sage species: A comparison between Salvia officinalis L., S. glutinosa L. and S. transsylvanica (Schur ex Griseb. & Schenk) Schur. Antioxidants 9(6) (2020) article 480, 1-19. https://doi.org/10.3390/antiox9060480.
[18] T. Belwal, P. Dhyani, I.D. Bhatt, R.S. Rawal, V. Pande, Optimization extraction conditions for improving phenolic content and antioxidant activity in Berberis asiatica fruits using response surface methodology (RSM), Food Chem. 207 (2016) 115–124, https://doi.org/10.1016/j.foodchem.2016.03.081.
[19] A.A. D'Archivio, M.A. Maggi, F. Ruggieri, Extraction of curcuminoids by using ethyl lactate and its optimisation by response surface methodology, J. Pharm. Biomed. Anal. 149 (2018) 89–95, https://doi.org/10.1016/j.jpba.2017.10.042.
[20] A. Antony, M. Farid, Effect of temperatures of polyphenols during extraction, Appl. Sci. 12 (4) (2022) 1–14, https://doi.org/10.3390/app12042107.
[21] N. Manousi, W. Wojnowski, J. Plotka-Wasylka, V. Samanidou, Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality, Green Chem. 19 (2023) 7598–7604, https://doi.org/10.1039/D3GC02347H.