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An Innovative Strategy to Enhance Polyphenol Content and Quality Traits of Olive Oil and Valorization of Mill Wastewater

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ABSTRACT

Olive oil polyphenols are a valuable help for human health and producing high-quality extra virgin olive oil rich in these polyphenols is a key objective for many farmers. In this work, we evaluated the impact of addition of salts to the milling process to increase the polyphenolic content of olive oil. The procedure proved successful, with efficacy dependent on the specific salt used and its concentration. The use of salts, in both laboratory and industrial settings, has increased not only the polyphenol content within the oil but also the yield and the qualitative–quantitative, sensory and nutritional characteristics of olive oil by impacting the amount of other minor constituents of olive oil, such as volatile compounds, chlorophyll, and carotenoids. Using the same approach, and by utilizing wastewater as a source of bioactives, we also successfully enriched other seed oils with olive oil polyphenols minimizing the waste of these substances and valorizing them. This enabled us to obtain a new class of seed oils enriched in antioxidant and anti-inflammatory compounds, potentially representing a new frontier in nutraceuticals.

1 | Introduction

Extra virgin olive oil (EVOO) is produced by cold pressing of the fruits (drupes) of the olive tree (*Olea europaea* L.) through a well-defined process governed by specific rules. According to the International Olive Council (IOC), EVOO is defined as "virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.80 g per 100 g and the other physico-chemical and organoleptic characteristics of which correspond to those fixed for this category in this standard." Approximately 98% of EVOO is composed of triglycerides, which constitute the *saponifiable fraction*, the majority of which (70%–85%) is esterified with monounsaturated or polyunsaturated fatty acids,

Abbreviations: EVOO, extra virgin olive oil; H_2Oveg, mill wastewater; HPP, high-pressure processing; HTy, hydroxytyrosol; MW, MicroWaves; Olea, oleacein; Oleo, oleocanthal; PEF, pulsed electric fields; SWIR, Visible and Short-Wave InfraRed; Ty, tyrosol; US, ultrasound.

Davide Laurenti and Daniel Di Risola contributed equally to this study.

such as oleic or linoleic acid, whereas the rest is represented by saturated fatty acids like palmitic or stearic acid (Jimenez-Lopez et al. 2020). The remaining 2%, the *unsaponifiable fraction*, is composed of tocopherols such as vitamin E, sterols, pigments like chlorophyll, aromatic and aliphatic alcohols, triterpenes, and polyphenols (Jimenez-Lopez et al. 2020).

The benefits of a diet including EVOO, such as the Mediterranean diet (Sofi et al. 2008), are widely described in the literature, and the effects of EVOO polyphenols against numerous degenerative diseases, including neurodegenerative, cardiovascular, and metabolic diseases, as well as cancer, are extensively demonstrated (Gorzynik-Debicka et al. 2018; Romani et al. 2019). Among the various polyphenols present in EVOO, Tyrosol (Ty), Hydroxytyrosol (HTy), Oleacein (Olea), Oleocanthal (Oleo), their respective precursors, Oleuropein aglycone and Ligstroside aglycone, and, albeit in lower concentrations, Oleuropein and Ligstroside, are those with the most documented bioactive effects and beneficial properties, particularly against oxidative stress and inflammation (Filardo et al. 2024; Pojero et al. 2022; Silvestrini et al. 2023).

Oleuropein aglycone and Ligstroside aglycone vary significantly depending on the cultivar, region, and production method of the EVOO (Losito et al. 2021). Both molecules possess marked antioxidant and anti-inflammatory properties. Specifically, oleuropein aglycone is known for its strong antioxidant activity, with the capacity to modulate oxidative stress and autophagy-related processes (Polacchini et al. 2025).

Ligstroside aglycone exhibits anti-inflammatory effects by modulating pathways such as NF-kB, MAPKs, JAK/STAT, Nrf2/HO-1, and the NLRP3 inflammasome, thereby reducing inflammation in LPS-stimulated macrophage cell models (Castejón et al. 2022). Furthermore, recent studies have demonstrated the ability of oleuropein aglycone to interact with proteins associated with pathological aggregation, such as α-synuclein, promoting the formation of nontoxic aggregates and reducing potential related cytotoxic processes (Palazzi et al. 2018). Additionally, Ligstroside aglycone is capable of suppressing tumor growth in vivo by modulating proliferation signaling pathways (Mahmud et al. 2025).

Overall, the beneficial effects of olive oil polyphenols are so well documented that EFSA authorizes the use of the following claim "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress." This claim may be used "... only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil. Information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil."

The concentration of polyphenols in EVOO, as well as the polyphenolic profile, can vary depending on several factors, such as cultivar, environmental conditions, harvesting and maturation stage of the drupes, oil extraction and production methods (Jimenez-Lopez et al. 2020).

The procedure for obtaining EVOO consists of seven essential phases: harvesting, washing, crushing, malaxation, extraction, storage, and packaging. Olives transported in specific containers are placed in a hopper where twigs and most leaves are removed. Subsequently, the olives are washed in the washing tank and dried before undergoing crushing, a phase in which they are mechanically broken using specific blades to obtain a regular particle size. The resulting olive paste is then placed in specific containers for the malaxation phase. This phase aims to break the water–oil emulsion and allow the oil micelles to coal into increasingly larger droplets, which tend to separate spontaneously from the water. During this phase some polyphenols, such as Oleuropein and Ligstroside, are converted by glucosidase into Oleuropein aglycone and Ligstroside aglycone, which are converted by the action of specific demethylases into Olea and Oleo, respectively, modifying the polyphenolic profile of the oil. Following the malaxation phase, extraction is carried out. The obtained paste is spread on filtering discs stacked to form a column and then pressed in a hydraulic press, which allows the extraction of a mixture of oil and water. An alternative method involves subjecting the olive paste to a centrifugation process inside a horizontal-axis rotating drum, known as a "decanter." Centrifugation separates the oil from the water due to their different specific gravities. However, this system requires the addition of a certain amount of water to the olive paste, leading to the removal of a significant quantity of polyphenols and affecting both the total content and the profile in the final product (Jimenez-Lopez et al. 2020). During the EVOO production process, waste products are generated, such as the solid residue (pomace) composed of skins, pulp, and fragments of the drupe's kernel, or the wastewater (H_2Oveg) rich in polyphenols and bioactive molecules. These waste products can pose an environmental problem if not properly managed, but can also represent potential added value if recovered through a circular economy of reuse (Batuecas et al. 2019). Pomace, for example, can be reused as biofuel or for the production of pellets, or used in agriculture as a soil improver or compost if treated (Romero-García et al. 2014; Černe et al. 2023). The behavior of olive mill wastewater in relation to the environment appears to be dualistic. On one hand, olive mill wastewater can be phytotoxic due to its high polyphenol content (Mekki et al. 2007) while, if appropriately diluted, can be used in agriculture as a fertilizer (Saf et al. 2023). Furthermore, polyphenols contained in H_2Oveg can exert antimicrobial activity in agriculture (Benguennouna et al. 2025).

Recently, innovative techniques have been developed to improve EVOO extraction to increase the yield, its stability, and the content of bioactives within the oil (M. Pérez et al. 2021; Peres et al. 2023). Pulsed electric fields (PEF), for instance, enhance oil extraction by increasing cell permeability and increase polyphenols and other bioactive compounds. High-pressure processing (HPP) increases the yield and oxidative stability of the product and can improve shelf-life and sensory quality. Ultrasound (US) promotes the breakdown of cell walls and shortens malaxation times, increasing yield and pigment content. Finally, MicroWaves (MW) reduce malaxation times, improve coalescence and yield, but can reduce the total polyphenol content or alter their profile. The fact that some of these solutions, such as the application of MW, are energy-intensive techniques with energy requirements 24% higher than others, can represent a negative element in the large-scale adoption of some of these emerging technologies. In the past, some researchers had already developed simple but effective ideas. Cruz et al. (2007) and Pérez et al. (2008), for example, were able to improve production yields and increase

phenolic compounds, β-carotene, and chlorophylls without compromising other fundamental parameters. Simply by adding sodium chloride (NaCl) at various concentrations to the olive paste. They tested the experimental hypothesis by using an experimental mini-mill (Abencor – Commercial Abengoa S.A., Seville, Spain), which efficiently reproduces the milling process on a reduced scale. Based on the intuition and the results obtained by these authors, we expanded the experimentation, investigating the impact of various salts used at different concentrations, up to their saturation point, to maximize the partitioning of bioactives between the water and oil phases. Subsequently, based on the results obtained in our laboratory, we extended the experimentation by using a non-experimental olive mill with the aim of performing an industrial scale-up.

Given the consistency between the laboratory results and those obtained during the industrial scale-up, which confirmed the feasibility of transferring polyphenols from an aqueous matrix to a lipid matrix by altering the partition coefficients, we applied the technique to develop nutraceutical seed oils enriched with polyphenols, reusing wastewater and potentially reducing its environmental impact.

2 | Materials and Methods

2.1 | Materials and Chemicals Used

The olives and the olive oil used for the analyses are the Coratina variety, purchased from the Maldera company (Maldera – Oleificio Sannicola s.r.l. – Via La Botte, 14 – 70033 Corato (BA)). The seed oil was purchased from Carapelli (Florence, Italy). For the preparation of saline solutions, NaCl was purchased from Sigma-Aldrich (Saint Louis, MO, United States), KCl from Carlo Erba (Cornaredo, MI, Italy), NaH_2PO_4 from VWR Chemicals (Geldenaakseban, Belgium), $CaCl_2$ and $NaNO_3$ from Merck (Darmstadt, Germany), and $MgCl_2$ and $(NH_4)_2SO_4$ were purchased from Fluka Chemika and Fluka Biochemika (Buchs, Switzerland), respectively. As for the reagents used for the chromatographic analyses, UPLC-grade water was purchased from VWR Chemicals, while UPLC-grade methanol and 98%–100% formic acid were purchased from Sigma-Aldrich (Darmstadt, Germany). The Folin & Ciocalteau reagent used for the analysis of total polyphenols was purchased from Sigma-Aldrich (Darmstadt, Germany). HTy, Ty, Olea, and Oleo standard molecules were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2 | Quantification of Polyphenols Repartition Between Water and Oil

For the quantification of HTy, Ty, Olea, and Oleo retained in the oil phase, 500 μL of olive oil were extracted with 500 μL of saline solution (or water as a control).

The salts used for this purpose were NaCl, $CaCl_2$, KCl, NaH_2PO_4, $MgCl_2$, $NaNO_3$ and $(NH_4)_2PO_4$. Concentrations of 1 M, 2 M and 4 M were tested for all of these salts. For NaCl, $CaCl_2$, $MgCl_2$, and $NaNO_3$, higher concentrations could be reached without precipitation, therefore the 6 M concentration was also tested.

The mixture of oil and saline solution/water was vortexed for 3 min and then centrifuged for 15 min at 14,000 rpm at 4°C. Subsequently, to extract and quantify the polyphenols still present in the oil, 200 μL of the oil phase were collected, and 200 μL MeOH/H_2O (80/20 ratio) were added. The resulting solution was vortexed for 1 min and then centrifuged for 5 min at 14,000 rpm at 4°C. The aqueous phase was collected and stored, and the extraction cycle was repeated two more times. All aqueous phases from the extraction cycles were pooled, vortexed for 1 min, and analyzed by UPLC/DAD/MS.

2.3 | Industrial Scale-Up

For the industrial scale-up, we collaborated with the Maldera company (Maldera – Oleificio Sannicola s.r.l. – Via La Botte, 14 – 70033 Corato (BA)), Frantoio La Valle dell'Usignolo di Giuseppe, Mario e Alfredo Palombo (Via Vigna Riccelli, 2 – 04013 Sermoneta (LT)), Frantoio Oleario Centro Agro Olivicolo Sonninese di Altobelli Angelo & C. (Via Argine Amaseno – 04010 Sonnino (LT)) which provided us with a three-phase olive mill. Before the extraction process, the olives were washed, and their water content was estimated using Visible (Vis) and Short-Wave InfraRed (SWIR) Spectroscopy. Based on the estimated water content within the drupes, the amount of NaCl to be added was calculated in order to reach a 4 M saline concentration during the malaxation phase. The salt was added 30 min after the beginning of malaxation to allow the enzymes to convert precursors such as Oleuropein aglycone and Ligstroside aglycone into oleacein and oleocanthal, respectively.

2.4 | Polyphenol Enrichment of Seed Oil

In order to obtain seed oils enriched with polyphenols derived from untreated olive mill wastewater (H_2Oveg), increasing concentrations of NaCl were added to the H_2Oveg. Subsequently, one volume of oil was mixed with one volume of H_2Oveg, vortexed, and centrifuged at 12,000*g* for 5 min to separate the oil and aqueous phases. The oil phase was then collected and analyzed for its polyphenol content. For the study on polyphenol transfer from H_2Oveg to seed oil as a function of the H_2Oveg/oil volume ratio, different amounts of H_2Oveg were used. Specifically, keeping the volume of seed oil constant, increasing volumes of H_2Oveg were applied (1 mL of seed oil, 100 μL of H_2Oveg: 10% water/oil ratio; 1 mL of seed oil, 200 μL of H_2Oveg: 20% water/oil ratio; 1 mL of seed oil, 400 μL of H_2Oveg: 40% water/oil ratio; 1 mL of seed oil, 800 μL of H_2Oveg: 80% water/oil ratio; 1 mL of seed oil, 1.5 mL of H_2Oveg: 150% water/oil ratio).

2.5 | Chromatographic Analyses

Chromatographic analyses were performed using a Waters Acquity UPLC system equipped with a quaternary solvent manager (QSM), a PDA detector set and a ESI–quadrupole mass detector (Waters, Milford, Massachusetts – 01757, USA). The analyses were carried out using a Kinetex C18 EVO column, 2.6 μm, 100 Å, 2.1 × 100 mm (Phenomenex, Torrance, CA, USA), thermostated at 35°C, and using the following gradient:

Minute	Flow (mL/min)	Solvent A (%)	Solvent B (%)
0	0.5	98	2
1	0.5	98	2
6	0.5	45	55
10	0.5	20	80
10.5	0.5	0	100
12.5	0.5	0	100
13	0.5	98	2
17	0.5	98	2

Solvent A consisted of water with 0.1% formic acid, and solvent B was methanol with 0.1% formic acid. As a control, a single-quadrupole mass detector with an electrospray ionization source (ACQUITY QDa) was also used to verify the exact m/z of the polyphenols.

2.6 | Total Polyphenol Content

For the determination of the total polyphenols present in the oil, a Folin–Ciocalteu assay was performed. Using a 24-well plate, 780 μL of water and 20 μL of the MeOH/H_2O 80/20 fraction used for polyphenol extraction from the oil were added to each well. Subsequently, 50 μL of Folin–Ciocalteu reagent were added, and the resulting solution was incubated in the dark for 2 min. After incubation, 150 μL of 20% Na_2CO_3 in water were added, and the reaction was incubated for 2 h in the dark. At the end of the reaction, the solution was analyzed using an UV-5100B UV/VIS spectrophotometer at a wavelength of 760 nm.

2.7 | Chlorophyll and Carotenoid Content

For the chlorophyll and carotenoid content, a spectrophotometric analysis was performed using a NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific – USA). In particular, 1 μL of each sample of olive oil was loaded on the pedestal of the nanodrop spectrophotometer and the spectrophotometric analysis was performed in the range 350–750 nm. The content was determined based on absorbance values (OD) of the oil measured at 670 and 470 nm, respectively, and applying the following conversion equations:

$$(OD_{670nm} \times 10) \times 10^6/61{,}300 \times e(OD_{470nm} \times 10) \times 10^6/200{,}000.$$

2.8 | Detailed Analysis of Oil Residues, Fatty Acids, and Volatile Compounds

The specific analysis of residues, fatty acid composition, and volatile compounds in the oil was carried out by a specialized and accredited company operating in accordance with UNI CEI EN ISO/IEC 17025 standards: Chemiservice S.r.l. – Via Vecchia Ospedale, Str. Priv. 11 – 70043, Monopoli (BA), Italy. The laboratory is recognized by the IOC for the physico-chemical analysis of olive oils and olive-pomace oils (Type A/B/C).

2.9 | Statistical Analysis

The software EXCEL and GraphPad Prism 8.0 were used to analyze and plot the data. The results are presented as mean ± standard deviation (SD). The analysis of statistical significance was performed using the unpaired *t*-test (the level of significance was set at $p < 0.05$) or by one-way ANOVA followed by a Tukey's post hoc test. Identical letters indicate that values are not significantly different, whereas different letters denote statistically significant differences between groups.

3 | Results

3.1 | Influence of Salts Addition on the Partitioning of Olive Oil Polyphenols Between Oil and Water

To investigate how specific salts affect the partitioning of EVOO polyphenols between water and oil phases, various salts—including NaCl, $CaCl_2$, KCl, NaH_2PO_4, $MgCl_2$, $NaNO_3$, and $(NH_4)_2SO_4$—were tested at concentrations up to their saturation points. All the used salts were nontoxic and commonly used as food ingredients or food additives (https://ec.europa.eu/food/food-feed-portal/screen/food-additives/search). Specifically, a volume of oil was mixed with a volume of H_2O containing the various salts at different concentrations, vortexed for 1 min, and centrifuged. After centrifugation, the oil phase was analyzed, and the polyphenol content was compared with that of oil samples mixed with equal volumes of H_2O without any salt. As expected, the mixing of the oil with H_2O and the subsequent steps depleted the oil phase of polyphenols, which were transferred to the aqueous phase. The percentage loss refers to the polyphenol amount that remains into water after extraction, in relation to the total polyphenol amount into the oil. In particular, the analysis performed by UPLC-DAD-MS highlighted a substantial disappearance of HTy (with a 98.59% loss), a reduction of Ty and of Olea (94.38% and 81.72%, respectively), and a 50.56% loss of Oleo from the oil, suggesting a relationship between the percentage loss and the hydrophobic characteristics of the molecule (Figure 1).

The addition of the various salts to the aqueous phase made it possible to reduce these percentage losses as a function of the salt concentration used. Indeed, as shown in Figure 1, the addition of each salt resulted in a lower transfer of polyphenols to the water phase, proportional to the increase in salt concentration up to the saturation point. The extent of the effect was different depending on the various salts used and was a direct consequence of the hydrophilic or hydrophobic nature of the various polyphenols (Supporting Information S1). Indeed, while the amount of polyphenol remaining in the oil phase was modest for HTy (ranging between 2.36% and 7.90%, depending on the concentration and the salt used), it became greater for Ty (9.40%–51.66%), for Olea (18.80%–85.55%), and Oleo (57.09%–97.11%). Among all the used salts, the one that showed the greatest ability to influence the partition coefficients was NaH_2PO_4, which, at a working concentration of 4 M, retained 7.90%, 46.81%, 85.55%, and 97.11% of HTy, Ty, Olea, and Oleo, respectively, in the oil phase. A similar behavior was evidenced using $(NH_4)_2SO_4$. To verify the behaviour of the different salts on the total polyphenol content, Folin–Ciocalteu assay was performed on the oil fractions treated as previously described. The analysis confirmed the greater ability of NaH_2PO_4 and $(NH_4)_2SO_4$ to retain a larger quantity of total polyphenols in the oil phase, followed by $CaCl_2$ and $MgCl_2$, and then by all the other salts. In particular, at a

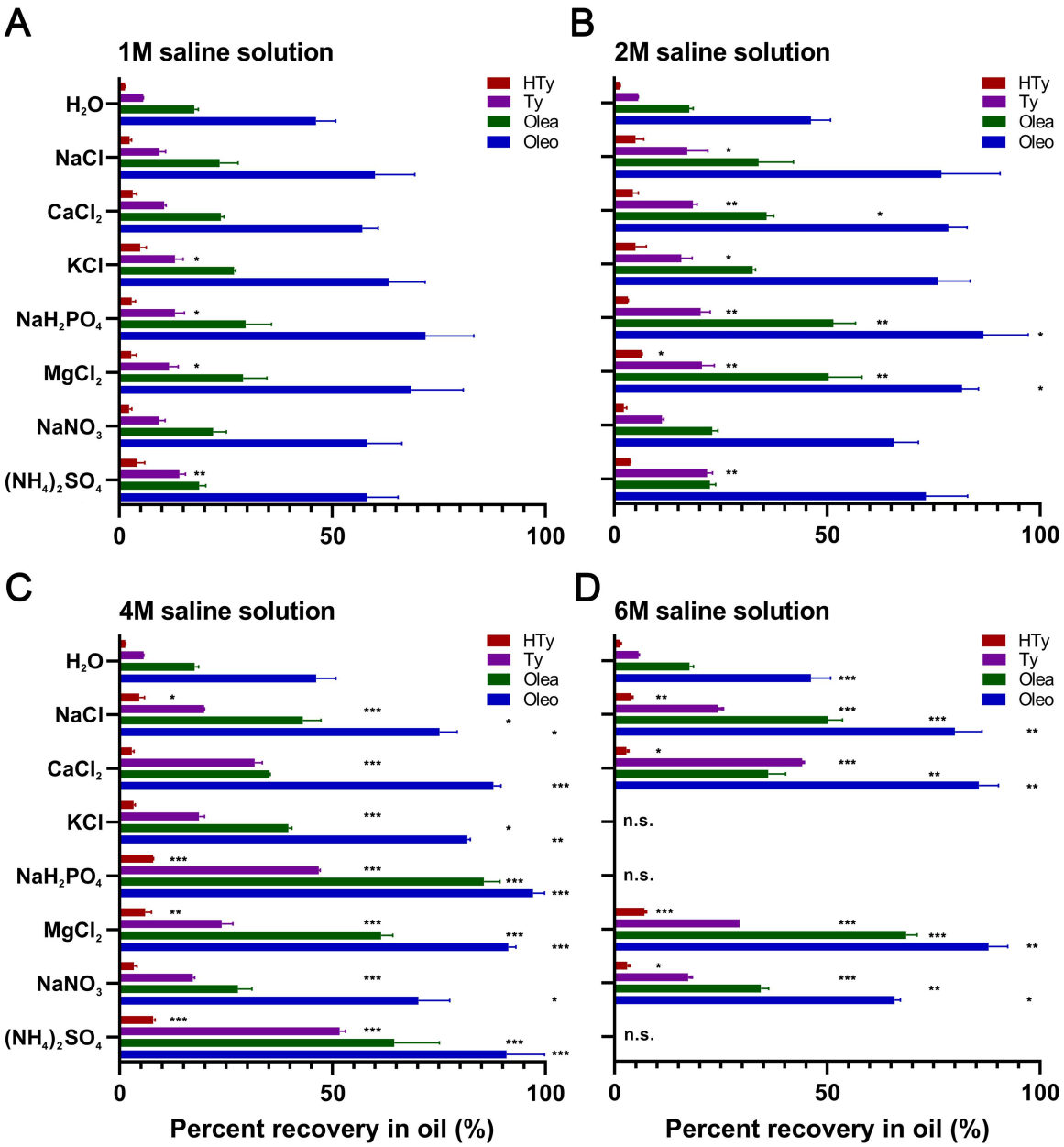

FIGURE 1 | Percentage recovery in the oil phase of individual polyphenols (HTy, Ty, Olea, Oleo) after mixing one volume of oil with one volume of water or water containing various salts at different concentrations (A) 1 M, (B) 2 M, (C) 4 M, (D) 6 M. Values are reported as means ± standard deviation (SD) of three independent samples. n.s., not soluble. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

concentration of 4 M, NaH_2PO_4 and $(NH_4)_2SO_4$ retained approximately 75% of the total polyphenols in the oil phase, while NaCl, KCl, and $NaNO_3$ retained about 50% (Figure 2).

These data indicate that all the tested salts, including NaCl, are able to influence the partition of the polyphenols present in EVOO between the water and oil phases, especially when used at a concentration close to their saturation point.

3.2 | Influence of Salt Addition During the Malaxation on Sensory and Nutritional Characteristics of Olive Oil

To determine the ideal conditions for performing an industrial scale-up in a nonexperimental mill, we based our decision on previous results. Considering both the extraction efficiency related to the type and concentration of salt, as well as the cost and availability of that salt, we decided to use 4 M NaCl. To this purpose, one oil mill located in Puglia region and two mills located in Lazio region were selected, and Coratina olives were chosen, a cultivar that already produces an oil characterized by a high polyphenol content and a high percentage of Olea and Oleo. To estimate the water content of the olives and accurately calculate the amount of NaCl to be added to reach a final concentration of 4 M we utilized a recently developed method which allows a rapid, nondestructive determination of the water content of the drupes by Visible and Short-Wave InfraRed (SWIR) spectroscopy (Bonifazi et al. 2024). The details of the developed Partial Least Squares regression model adopted to estimate the water content of the olives are reported in

Supporting Information S1. The determination of the water content in the olive drupes is necessary to accurately estimate the amount of NaCl to be added during the malaxation process. This is required to maintain the 4 M salt concentration in the solution and optimize the partition coefficient of polyphenols into the olive oil.

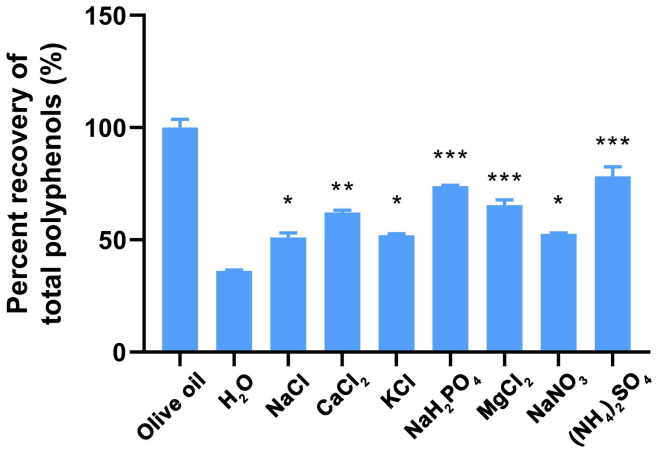

FIGURE 2 | Percentage recovery of total polyphenols after mixing one volume of oil with one volume of water or water containing various salts at 4 M concentration. Values are reported as means ± standard deviation (SD) of three independent samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

When NaCl was added during the malaxation phase, and in particular 30 min after its start, the total polyphenol concentration, expressed as HTy equivalents, increased significantly from 815.16 ± 16.40 mg/kg of olive oil to 1265.92 ± 35.15 g/kg (Figure 3A). The polyphenolic profile also changed significantly. In fact, while the amounts of HTy and Ty in the sample treated with NaCl remained similar to those in the control sample, the amounts of Olea and Oleo increased from 169.44 ± 7.06 mg/kg to 457.77 ± 19.08 mg/kg of olive oil and from 383.58 ± 15.98 mg/kg to 464.36 ± 19.35 mg/kg of olive oil, respectively (Figure 3B). The oil extraction efficiency also increased, improving yields by approximately 10%. Apart from improving the oil yield, the total polyphenol content, and particularly the levels of Olea and Oleo, the addition of NaCl also increased the amounts of chlorophyll and carotenoids, as shown in Figure 3C,D. Specifically, the spectrophotometric profile revealed an enrichment in the NaCl-treated sample compared to the control sample, with chlorophyll and carotenoid levels increased by 3 and 2.3 times, respectively. Finally, by an extensive analysis aimed at evaluating the qualitative and organoleptic characteristics of the olive oil produced according to the described procedure, it emerged that the technique does not alter the fatty acid composition of the oil (Table 1) but

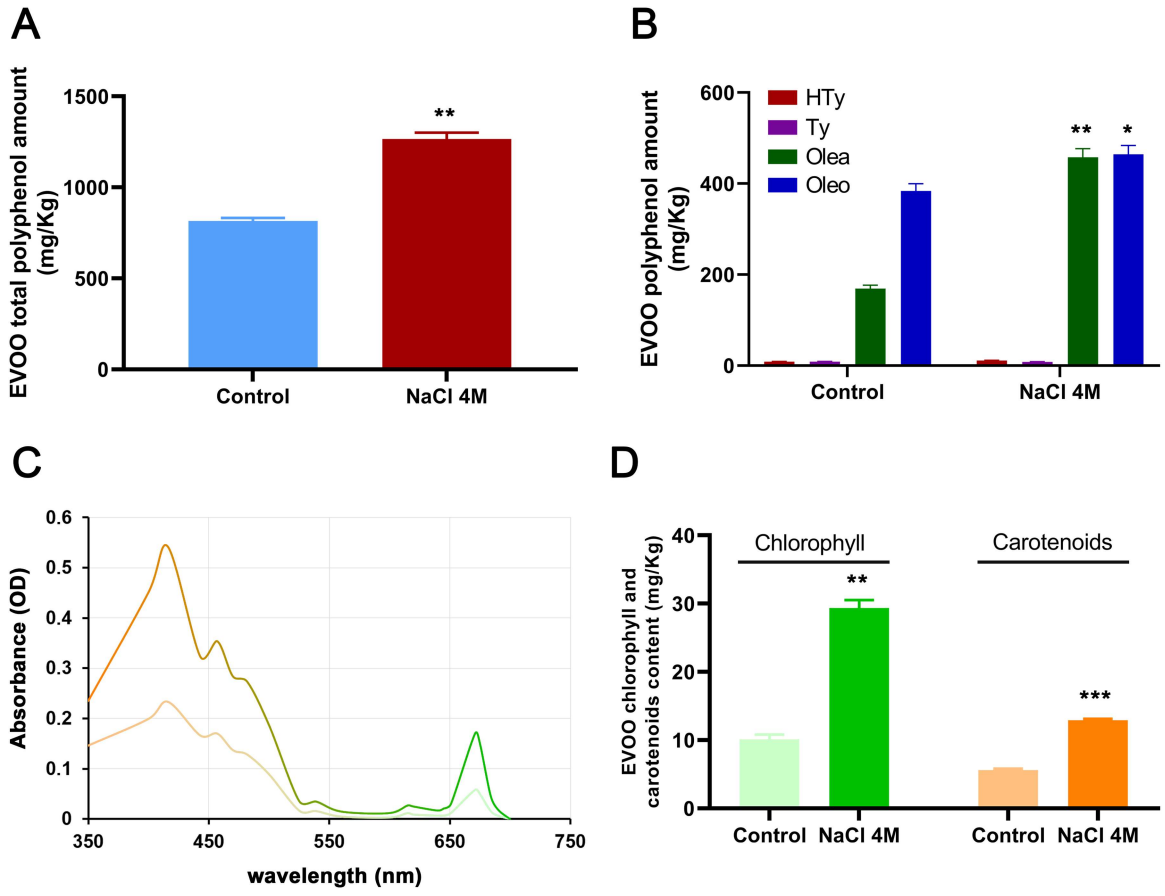

FIGURE 3 | Analysis of oil obtained after treatment with 4 M NaCl in an industrial facility, compared with an untreated control. (A) Total polyphenol content (mg/kg). (B) Content of individual polyphenols (HTy, Ty, Olea, Oleo) (mg/kg). (C) Absorption spectrum in the VIS range 350–750 nm (green: chlorophyll; orange: carotenoids). (D) Quantification of chlorophyll and carotenoids (mg/kg). All values are expressed as means ± standard deviation (SD) of two independent samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

TABLE 1 | Fatty acid content in control oil and in oil obtained by NaCl treatment.

Alkyl esters (methyl and ethyl esters of fatty acids)	Control ± (analytic error)	NaCl ± (analytic error)	*s* (propagated error)	CI (95%) |Z| > 2
Total methyl esters (mg/kg)	1 ± 1	1 ± 1	1.41	0.00
Total ethyl esters (mg/kg)				
Total methyl and ethyl esters (mg/kg)	1 ± 1	1 ± 1	1.41	0.00
Methyl esters of fatty acids (%)				
C14:0 – Myristic acid	0.01 ± 0.01	0.01 ± 0.01	0.01	0.00
C16:0 – Palmitic acid	9.53 ± 0.54	9.61 ± 0.55	0.77	0.10
C16:1 – Palmitoleic acid	0.47 ± 0.04	0.45 ± 0.04	0.06	0.35
C17:0 – Heptadecanoic acid	0.05 ± 0.01	0.04 ± 0.01	0.01	0.71
C17:1 – Heptadecenoic acid	0.06 ± 0.01	0.06 ± 0.01	0.01	0.00
C18:0 – Stearic acid	2.68 ± 0.18	2.65 ± 0.18	0.25	0.12
C18:1 – Oleic acid	78.89 ± 3.27	78.89 ± 3.27	4.62	0.00
C18:2 – Linoleic acid	6.52 ± 0.39	6.56 ± 0.40	0.56	0.07
C20:0 – Arachidic acid	0.47 ± 0.04	0.45 ± 0.04	0.06	0.35
C18:3 – Linolenic acid	0.74 ± 0.06	0.73 ± 0.06	0.08	0.12
C20:1 – Eicosenoic acid	0.39 ± 0.04	0.37 ± 0.03	0.05	0.40
C22:0 – Behenic acid	0.13 ± 0.01	0.13 ± 0.01	0.01	0.00
C22:1 – Erucic acid	n.d.	n.d.		
C24:0 – Lignoceric acid	0.06 ± 0.01	0.05 ± 0.01	0.01	0.71
Trans isomers of fatty acids (%)	0.02 ± 0.01	0.02 ± 0.01	0.01	0.00
C18:1 – (Elaidic acid)	0.01 ± 0.01	0.01 ± 0.01	0.01	0.00
C18:2 + C18:3	n.d.	n.d.		

rather impacts the amount of volatile compounds, altering, for example, 2-decenal, 2-heptenal, octanal, and so forth (Table 2).

3.3 | Transfer of Polyphenols From Olive Oil Mill Wastewater to Seed Oil

Given the results reported above, we attempted to extend the application by using olive mill wastewater (H_2Oveg), produced during the EVOO production processes, which is extremely rich in polyphenols, to transfer the bioactive molecules contained therein into any lipid matrix, such as seed oils.

To this purpose, corn seed oil was used, and NaCl was added to the H_2Oveg at various concentrations. Different H_2Oveg/oil volume ratios were used. The analysis using Folin–Ciocalteu, employed to quantify the transfer of total polyphenols from the H_2Oveg to the oil, highlighted that as the salt concentration increased, the amount of total polyphenols transferred progressively increased up to the saturation point (6 M) (Figure 4A). Using this latter NaCl concentration and keeping the volume of seed oil constant, increasing volumes of H_2Oveg were applied (details are provided in Section 2) and the results, shown in Figure 4B, were obtained. The experimental data showed that even a small H_2Oveg volume (10%) is sufficient to transfer a large amount of polyphenols, and this value increases with increasing H_2Oveg/oil volume ratios used. In particular, when compared with the starting seed oil sample, which is obviously devoid of polyphenols (data not shown), the addition of 10% H_2Oveg yields a seed oil with a total polyphenol concentration of 809.69 ± 17.95 mg/kg of oil. The amount of total polyphenols tends to increase nonlinearly in the other samples, reaching a value of 1495.37 ± 17.96 mg/kg in the last sample (Figure 4B).

The analysis of the polyphenolic profiles, performed by UPLC-DAD-MS, highlighted that the more hydrophilic polyphenols HTy and Ty were found in the different tested samples at low concentrations ranging between 0.88–1.24 mg/kg and 2.39-4.43 mg/kg, respectively. Furthermore, while HTy levels did not increase significantly with H_2Oveg/oil volume ratios higher than 20%, Ty levels showed increases in all samples with a linear trend (Figure 5A,B).

Regarding Olea and Oleo, the chromatographic analysis highlighted their high concentrations in all tested samples, with the obvious exception of the control sample. In particular, Olea concentrations ranged between 598.02 mg/kg and 850.61 mg/kg, while Oleo concentrations ranged between 79.78 mg/kg and 244.93 mg/kg. Interestingly, similar trends to those observed for HTy and Ty were also recorded for Olea and Oleo. Specifically, while Olea levels increased by approximately 20% when moving from H_2Oveg/oil volume ratios of 10% to H_2Oveg/oil volume ratios of 40% and remained stable in all other samples, Oleo levels showed significant increases in all tested samples according to a linear progression, increasing from 79.78 mg/kg to 244.93 mg/kg in the last sample (Figure 5C,D).

TABLE 2 | Volatile compounds content in control oil and in oil obtained by NaCl treatment.

Volatile compounds (mg/kg)	Control ± (analytic error)	NaCl ± (analytic error)	*s* (propagated error)	CI (95%) |Z| > 2
1-Hexanol	0.67 ± 0.17	0.44 ± 0.11	0.20	1.14
1-Octen-3-ol	n.d.	0.04 ± 0.01		
1-Penten-3-ol	0.39 ± 0.1	0.62 ± 0.16	0.19	1.22
2-Methyl-1-butanol	n.d.	n.d.		
2-Octanol	n.d.	n.d.		
2-Octanone	n.d.	n.d.		
3-Methyl-1-butanol	n.d.	n.d.		
4-Ethyl phenol	0.05 ± 0.01	0.05 ± 0.01	0.01	0.00
4-Ethylguaiacol	n.d.	n.d.		
6-Methyl-5-hepten-2-one	n.d.	0.04 ± 0.01		
2-Decenal	0.50 ± 0.13	1.19 ± 0.30	0.33	2.11
2-Heptenal	0.04 ± 0.01	0.21 ± 0.05	0.05	3.33
2-Hexenal	15.69 ± 3.92	30.75 ± 7.69	8.63	1.74
2-Hexenol	0.44 ± 0.11	0.3 ± 0.08	0.14	1.03
2-Nonenal	0.01 ± 0.01	0.02 ± 0.01	0.01	0.71
2-Octenal	0.02 ± 0.01	0.02 ± 0.01	0.01	0.00
2-Pentenal	0.06 ± 0.02	0.06 ± 0.02	0.03	0.00
2.4-Hexadienal	0.55 ± 0.14	0.41 ± 0.1	0.17	0.81
2-Pentenol	0.57 ± 0.14	0.69 ± 0.17	0.22	0.54
3-Hexenol	0.76 ± 0.19	0.68 ± 0.17	0.25	0.31
3-Hexenyl acetate	0.27 ± 0.07	0.26 ± 0.07	0.10	0.10
Acetic acid	0.83 ± 0.21	1.22 ± 0.31	0.37	1.04
Butanoic acid	n.d.	0.02 ± 0.01		
Ethanol	2.29 ± 0.57	1.84 ± 0.46	0.73	0.61
Ethyl 2-methyl butanoate	n.d.	n.d.		
Ethyl acetate	0.03 ± 0.01	0.04 ± 0.01	0.01	0.71
Ethyl butanoate	n.d.	n.d.		
Ethyl hexanoate	n.d.	n.d.		
Ethyl propanoate	n.d.	n.d.		
Ethyl vinyl ketone (1-penten-3-one)	1.40 ± 0.35	1.66 ± 0.42	0.55	0.48
Guaiacol	n.d.	n.d.		
Hexanal	0.33 ± 0.08	0.36 ± 0.09	0.12	0.25
Hexanoic acid	1.92 ± 0.48	3.16 ± 0.79	0.92	1.34
Hexyl acetate	n.d.	n.d.		
Nonanal	0.47 ± 0.12	0.45 ± 0.11	0.16	0.12
Octanal	0.05 ± 0.01	0.2 ± 0.05	0.05	2.94
Octane	0.21 ± 0.05	0.25 ± 0.06	0.08	0.51
Pentanal	0.11 ± 0.03	0.27 ± 0.07	0.08	2.10
Pentanoic acid	0.09 ± 0.02	0.2 ± 0.05	0.05	2.04
Propanoic acid	n.d.	n.d.		

4 | Discussion

In recent years, the interest in olive oils rich in polyphenolic compounds has gained more and more attention due to their potential utilization as nutraceutical products to counteract degenerative diseases and cancer (Filardo et al. 2024).

Among the different factors that may influence the polyphenol content in EVOO, that is the degree of olive ripening, cultivar, climatic conditions, and technology used for the production, this latter represents the one that can be better controlled and manipulated under standardized conditions to obtain enriched

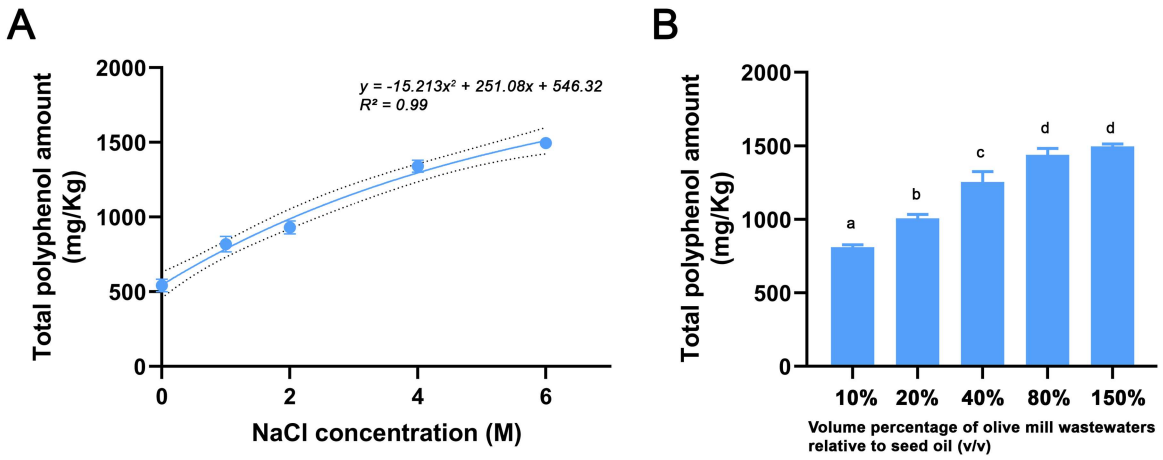

FIGURE 4 | Analysis of polyphenols transferred from the olive mill wastewater to the seed oil. (A) Total polyphenol amount transferred from the olive mill wastewater to the seed oil depending on NaCl concentration. (B) Total polyphenol amount transferred from the olive mill wastewater to the seed oil depending on volume percentage of olive mill wastewater relative to seed oil (v/v), using NaCl at 6 M concentration. All values are expressed as means ± standard deviation (SD) of two independent samples. The letters above each histogram indicate statistically distinct groups.

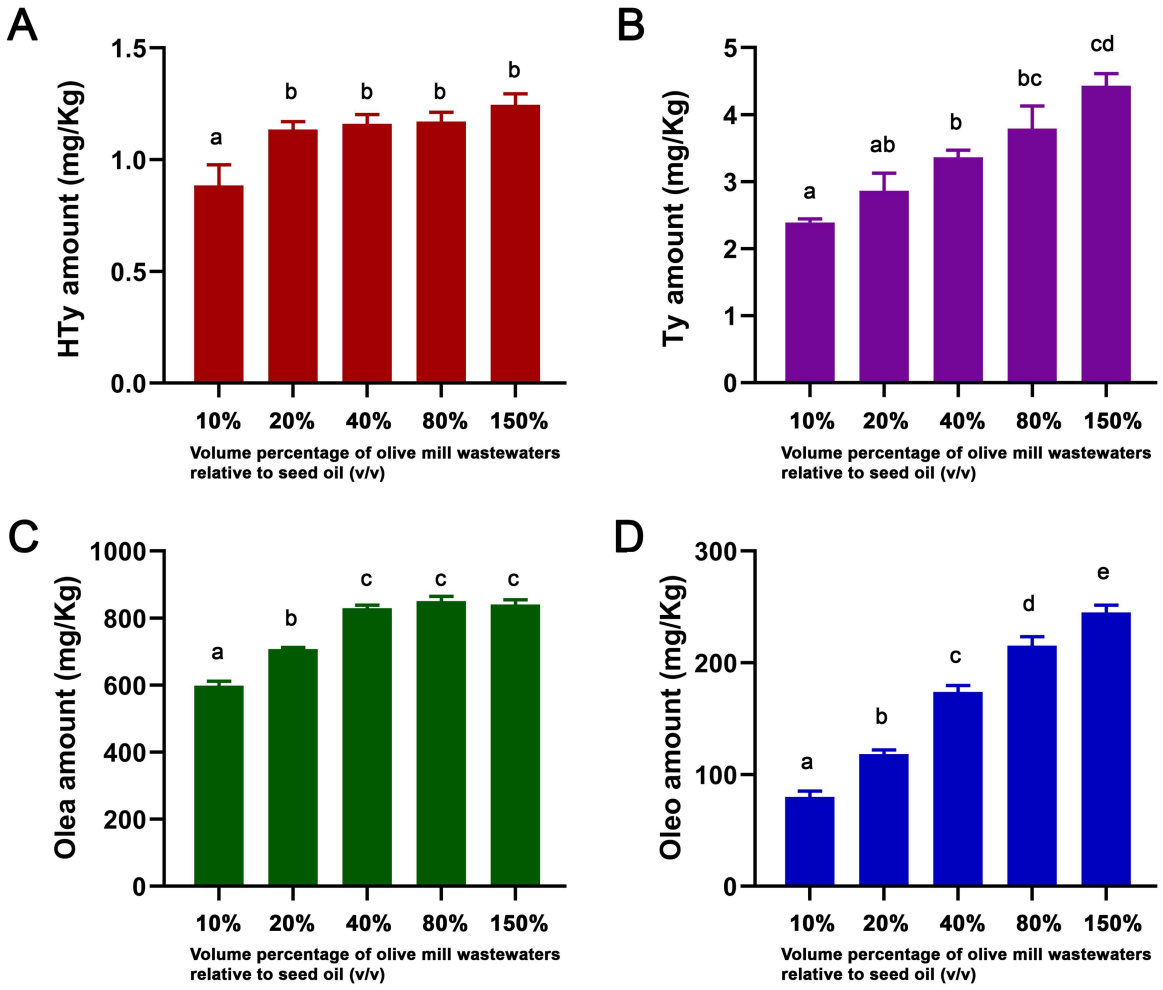

FIGURE 5 | Analysis of single polyphenols (A) HTy, (B) Ty, (C) Olea, (D) Oleo, transferred from the olive mill wastewater to the seed oil depending on volume percentage of olive mill wastewater relative to seed oil (v/v), using NaCl at 6 M concentration. All values are expressed as means ± standard deviation (SD). The letters above each histogram indicate statistically distinct groups.

oils. The technique based on PEF, tested on different cultivars, is able to increase oil yields by up to 18%, slightly increases (3.2%–14.3%) the total polyphenol content, including Olea and Oleo, and improves oxidative stability while leaving the sensory profile unaltered (Leone et al. 2022). Similar results are obtained by applying the HPP technique with a 16% increase in oil yield and improved oxidative stability (M. Pérez et al. 2021). No data are reported regarding variations in polyphenol content. Better extraction efficiency is also obtained through the application of US, which promotes the breakdown of cell walls, shortens malaxation times, and increases pigments such as tocopherols, chlorophylls, and carotenoids, whereas conflicting results are obtained regarding variations in polyphenolic profiles (Servili et al. 2019). Finally, the use of MW is able to increase yields by up to 5.4% while reducing malaxation times but has a negative impact on the total polyphenol content (M. Pérez et al. 2021). Overall, all these promising and innovative applications offer significant advantages such as increased oil yields or the reduction of wastes, but they imply the use of additional instrumentation, often technologically complex or energy-intensive, as in the case of MW.

In the aim of increasing olive oil polyphenol content, various attempts to modulate factors of the malaxation process have been made, including timing of the process, temperature, limitation of the use of added water, addition of enzymes such as cellulases, and finally the use of additives such as salts or talc (Koprivnjak et al. 2016; Majetić Germek et al. 2016). A few reports in the literature described the use of sodium chloride as a mean to transfer polyphenols from the water phase to the oil (A. G. Pérez et al. 2008; Koprivnjak et al. 2016; Majetić Germek et al. 2016).

The use of NaCl represents an interesting low-cost and easily applicable alternative method. The principle behind its use lies in the creation of an osmotic gradient that causes dehydration of the drupe cells. This process facilitates the rupture of cell membranes and the release of water-soluble compounds such as polyphenols, improving extraction efficiency. The addition of NaCl increases the ionic strength of the aqueous phase, triggering a "salting-out" effect. This phenomenon reduces the solubility of amphiphilic compounds, such as polyphenols, in water. The aqueous phase becomes less favorable for organic solutes and the partition coefficient shifts in favor of the lipid phase. Consequently, a higher concentration of bioactive phenols is retained in the oil. In addition to the osmotic effect, salt could exert a direct influence on enzymatic activity. It is known that enzymes such as polyphenol oxidase are responsible for the degradation of polyphenols during olive processing (Taticchi et al. 2013). Highly concentrated sodium chloride could inhibit their activity, thus limiting phenolic oxidation and thus helping to preserve the nutritional and sensory quality of olive oil.

In comparison to more advanced emerging technologies, the use of salt presents notable advantages in terms of operational ease. It does not require specialized equipment or advanced technical expertise and can be seamlessly integrated into conventional crushing systems. Moreover, it has minimal environmental impact, generates no chemical residues, and does not lead to increased energy consumption.

Overall, our data corroborate those previously obtained by A. G. Perez et al. (2008), demonstrating that the addition of NaCl during the olive oil extraction process increases the transfer of polyphenols to the oil phase, producing high added-value oils with nutraceutical potential. Furthermore, as evidenced by the data presented in this manuscript, the technique appears to be applicable and well-suited to large, nonexperimental oil mills, and therefore easily scalable. In addition to the differences in the oil mill (experimental vs. nonexperimental) and the drupe cultivar (Picual vs. Coratina) used, the substantial differences between Perez's experimental setup and that presented in this work, concern the NaCl concentrations used and the presence/absence of talc. This could explain at least in part the slight differences observed in some data between the two studies. In particular, according to Perez et al. the levels of more hydrophilic polyphenols such as HTy and Ty decrease in a statistically significant manner even with a 1% NaCl concentration. The values of Olea and Oleo also decrease progressively and significantly with increasing NaCl concentration. In contrast, our data do not show a decrease in HTy, Ty, Olea, and Oleo levels. On the other hand, although HTy and Ty levels remain stable, Olea and Oleo levels increase relative to the control. It should be emphasized, however, that in both Perez's work and the present manuscript, the quantities of HTy and Ty are dozens of times lower than those of Olea and Oleo, contributing only minimally to the total polyphenol concentration in olive oil. The concentration of total polyphenols, as well as their profile, can vary depending on many factors (Jimenez-Lopez et al. 2020), but it is important to highlight that EVOO with high levels of Olea and Oleo are receiving particular attention from the scientific community and consumers for the beneficial properties exerted by these two polyphenols.

Indeed, both Olea and Oleo have demonstrated antioxidant, anti-inflammatory, antitumor, and antibacterial activities, and protective properties against neurodegenerative and cardiovascular diseases (Di Pietro et al. 2024; Filardo et al. 2024; Kusuma et al. 2024; Castejón et al. 2020). In particular, Olea and Oleo are known for their ability to inhibit cyclooxygenases (COXs), enzymes involved in the synthesis of prostaglandins, mediators of inflammation (Ricciotti and Fitzgerald 2011), exerting an action similar to or greater than that of ibuprofen (Di Risola et al. 2025). Furthermore, Olea and Oleo are able to modulate both in vitro and in vivo the expression of genes encoding for pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α (Cirmi et al. 2022; Scotece et al. 2012) and to interfere with the Nrf2 and NF-κB signaling pathways (Cirmi et al. 2022; Carpi et al. 2019; Filipek and Gierlikowska 2021; Montoya et al. 2019). A recent systematic review summarized the mechanisms through which Olea and Oleo exert chemoprotective effects (Kusuma et al. 2024). They induce apoptosis and inhibit cell proliferation, angiogenesis, migration, and metastasis in models of breast, prostate, liver, lung, and pancreatic cancer. They interfere with crucial pathways such as mTOR, AKT, ERK1/2, STAT3 (Cirmi et al. 2020), and modulate the expression of some miRNAs involved in tumor progression such as miR-193a-5p, miR-34a, miR-16. At the epigenetic level, they inhibit the activity of histone deacetylases, promoting antitumor profiles. Also, with regard to neurodegenerative diseases, thanks to their anti-inflammatory properties and their ability to reduce oxidative stress, reactive oxygen and nitrogen species, Olea and Oleo contribute to protection against diseases such as Alzheimer, Parkinson, and Amyotrophic Lateral Sclerosis (Gonçalves et al. 2024). Moreover, Olea has been

shown to upregulate the ACHE and BACE2 genes in an in vitro model of Alzheimer's disease (Polerà et al. 2026), as well as to prevent high-fat-diet–induced adiposity and improve certain biochemical parameters of insulin sensitivity in mice (Lepore et al. 2019).

It is therefore not surprising that great efforts are directed toward the study and development of techniques that allow obtaining olive oil particularly rich in Olea and Oleo without compromising fundamental characteristics such as the lipid profile, acidity, or organoleptic properties. In addition to the polyphenolic profile, some important volatile compounds also appear to be altered by the process described in this manuscript. Indeed, Koprivnjak et al. (2016) had already described changes in the volatile compound profile of oil obtained from drupes of the Croatian cultivar Buža, using NaCl, talc, or their combinations during the production process. In particular, the researchers found that the addition of NaCl does not alter the C6 aldehyde compounds (reported as the sum of E-2-hexenal, hexanal, (E,E)- or (E,Z)-2,4-hexadienal, Z-3-hexenal), which are crucial for the characteristic fresh and fruity aroma of the oil, but tends to increase the corresponding C6 alcohols (reported as the sum of Z-3-hexen-1-ol, 1-hexanol, E-3-hexen-1-ol, E-2-hexen-1-ol). The effect of NaCl on the increase of terpenes and C5 compounds contributing to the aromatic complexity is also noteworthy. Although a direct comparison between the data reported by Koprivnjak and colleagues and those obtained by us in the present manuscript is not possible, an important, albeit not significant, increase in 2-hexenal and no change in 1-hexanol levels should be highlighted. These data suggest some differences between the results reported by Koprivnjak et al. and those presented in this manuscript, differences that could derive from the cultivars used (Buža vs. Coratina) or some details in the application of the technique, such as the use of industrial-scale equipment rather than experimental setups (e.g., Abencor), which could also explain the discrepancies, compared to other studies, in polyphenol content and composition described above.

As we have experienced, NaCl is not the only usable salt with concrete effectiveness, in fact $CaCl_2$, KCl, NaH_2PO_4, $MgCl_2$, $NaNO_3$, and $(NH_4)_2SO_4$ have also proved to be highly efficient. The reduction of the polyphenol content in wastewater, guaranteed by the described technique, is an essential condition to avoid negative and phytotoxic effects resulting from an excess of polyphenols (Mekki et al. 2007). The presence of various salts, moreover, could make the product suitable for fertilization and provide the right amount of potassium (KCl) (Zörb et al. 2014), nitrogen ($NaNO_3$, $(NH_4)_2SO_4$) (Fowler et al. 2013), and phosphorus (NaH_2PO_4) (Ahmad et al. 2023), could be applied as a foliar fertilizer ($MgCl_2$) (Zhang et al. 2022), could be used as an antifeeding agent ($CaCl_2$) (Chakraborty et al. 2017; Wang et al. 2021) or as a soil amendment ($CaCl_2$, $(NH_4)_2SO_4$) (Gao et al. 2015; Chien et al. 2011), improving the current applications of wastewater within a circular economy with a lower environmental impact. However, we acknowledge that, at the current state of the art, the discussion on potential agricultural applications of salt-laden wastewater as a source of potassium, nitrogen, or phosphorus is entirely speculative and requires specific experimental investigation and validation.

Finally, by extending the technique, here we demonstrated that salt-enriched wastewater can be used to fortify seed oils such as sunflower and corn oil, or any other lipid matrix, with polyphenols. This approach could enable the production of high added-value nutraceutical oils or fats, with potential for commercialization even in countries where olive oil is not traditionally widely consumed.

5 | Conclusion

Overall, all the data demonstrate that, by adding salts appropriately selected based on their effectiveness, it is possible to modulate the partition coefficient of polyphenols between the water and oil phases, both under laboratory conditions and in nonexperimental olive mills, enhancing the amount of polyphenols in the olive oil. This result is strictly influenced by the type of salt used and by its concentration. Moreover, the use of salts during olive oil production allows the retention of other chemical species, such as chlorophyll and carotenoids, which increase significantly compared to the standard olive oil production method and modify the levels of some volatile compounds that confer to the olive oil specific taste and flavor.

Finally, this strategy can be exploited to transfer the polyphenols present in olive oil mill wastewater to other lipid matrices, such as seed oil, allowing a recycling of wastewater. All in all, it is possible not only to enhance the polyphenol content and quality traits of olive oil but also to valorize the olive oil mill wastewater.

Author Contributions

Davide Laurenti: methodology, investigation, data curation. **Daniel Di Risola:** methodology, investigation, data curation, writing – review and editing. **Antonio Francioso:** methodology, investigation, data curation. **Rodolfo Federico:** conceptualization, funding acquisition. **Eugenio Lendaro:** conceptualization and supervision. **Riccardo Gasbarrone:** methodology, investigation, data curation. **Giuseppe Bonifazi:** conceptualization, supervision. **Mario Fontana:** conceptualization, supervision. **Luciana Mosca:** conceptualization, funding acquisition, resources, supervision, writing – review and editing. **Roberto Mattioli:** conceptualization, methodology, data curation, writing – original draft.

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Conflicts of Interest

Rodolfo Federico is a founding member of the Company Active-Italia S.r.l. Luciana Mosca, Antonio Francioso, Roberto Mattioli and Rodolfo

Federico are inventors named on the patent titled "Metodo per la preparazione di olio di oliva con caratteristiche qualitative, sensoriali e nutrizionali determinate," Patent No. IT102023000006075, filed by Active-Italia S.r.l. Roma (IT). Luciana Mosca, Roberto Mattioli and Rodolfo Federico are inventors named on the patent titled "Metodo per la preparazione di matrici oleose e/o lipidiche arricchite in polifenoli," Patent No. IT102024000017041, filed by Active-Italia S.r.l. Roma (IT). The remaining authors declare no competing financial interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Chromatogram of hydroxytyrosol (a), tyrosol (b), oleacein (c) and oleocanthal (d) performed on C18 column, whose retention times are in agreement with their CLogP values estimated by ChemDraw software.

Figure S2: Raw reflectance (a) and pre-processed spectra (b) of the olive fruits according to water content.

Figure S3: PCA scores plot for water content of the first and second Principal Components (PCs) (a) and loadings plot of the first PC (b).

Figure S4: Comparison of PLS regression for water content performed in the spectral regions 400–2500 nm (a) and the VIP scores resulting from PLS regression (b).

Table S1: Statistical parameters of the performed PLS regression.