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RESEARCH ARTICLE

Extracellular vesicles from olive wastewater and pomace by-products: Isolation, characterization of their lipid and phenolic profiles, and evaluation of their radical scavenging activity

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Abstract

Extracellular vesicles (EVs) are nanoscopic structures that are involved in intercellular communication. Recent works have highlighted the existence of these assemblies in several plants and shown that they are able to vectorize hydrophilic and lipophilic molecules. In this study, we have isolated EVs from the two main olive oil by-products (wastewaters [WWs] and pomace) by differential centrifugation/ultracentrifugation and have characterized their main physicochemical properties (size, charge, multimolecular structure, lipid and phenolic contents) and radical scavenging activity. Lipid content in EV fractions was 3.4 (0.2) % (% dry material) for WWEVs and 7.7 (0.3) % and 5.9 (0.9) % for EVs, respectively, from plurivarietal or monovarietal pomaces. Polar lipids represented around 49% of total lipids, and their profiles were globally similar in all EVs. Phosphatidylcholine and phosphatidic acid were the more abundant molecules. Their phenolic contents ranged from 2.1 to 4.6 mg hydroxytyrosol (HT) eq g^{-1} of raw material, with HT, oleuropein, and verbascoside being among the most abundant. Transmission electron cryomicroscopy showed the presence of

Abbreviations: ALA, α-linolenic acid; CryoTEM, transmission electron cryomicroscopy; DAG, diolein; DGDG, digalactosyldiacylglycerol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EV, extracellular vesicles; FAME, fatty acid methyl esters; FFA, oleic acid; HT, hydroxytyrosol; MAG, 1-monoolein; MGDG, monogalactosyldiacylglycerol; OL, oleuropein; PA, 3-phosphatidic acid; PBS, phosphate buffer solution, pH 7.2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TAG, triolein; VB, verbascoside.

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spherical vesicles delimited by a single bilayer of amphiphilic lipids. Finally, the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of EVs was high and depended on their original by-product type.

Practical Application: Recent works have highlighted the existence of extracellular vesicles in several plants and shown that they are able to vectorize hydrophilic and lipophilic molecules. Herein, we have isolated and provided a chemical characterization of such vesicles from olive wastewater and pomace. Results showed that these vesicles are rich in the phenolic compounds that are generally found in olives and that the potential radical scavenging activity of extracellular vesicles from olive could be valorized as new antioxidants for the food or cosmetic sectors.

KEYWORDS
antioxidants, extracellular vesicles, olive by-products, phenolic compounds, polar lipids

1 | INTRODUCTION

Extracellular vesicles (EVs) are nanoscopic membranous structures that are secreted by diverse cells and are involved in intercellular communications as vehicles of various molecules, including proteins, nucleic acids (including small RNAs), and lipids. Accordingly, the effects of EV have been extensively studied, especially their impact on the progression of various diseases, in particular cancer [1–4]. Moreover, as EVs are nature's tools for intercellular communications, they were also recently investigated as natural drug delivery systems and compared with artificial nanoparticles such as liposomes [5–7]. In that context, EVs may present some advantages with the possibility of being engineered to deliver their cargo more specifically to the targeted sites, thereby minimizing the systemic effect [8]. To date, EVs are mainly described and characterized for mammalian cells. There are indeed encountered in many diverse biofluids, such as blood, saliva, urine, brain fluids, or milk. However, EVs were also identified in bacteria or fungi, protozoa, and plants [9].

Plants have recently been shown to release EV into cell apoplast [10], and such vesicles have been visualized in various plants, such as barley leaf [11], citrus [12], carrot, ginger [13], sunflower [14], grape [15], or tea flowers [16]. Beside their role in intercellular communications inside the concerned plant, plant EVs are also believed to allow small RNA trafficking between plant hosts and pathogens [17]. Plant EVs also contain antimicrobial compounds and defense-related proteins and are able to deliver this cargo to invading fungi [18].

EVs are unilamellar and nanoscopic, usually in the range of 150–300 nm, but sometimes are smaller (50–110 nm). Moreover, the concomitant presence of polyphenolic species in plant EV can be advantageous for the conception of new bioactive delivery systems with potential applications in nutrition and health. Indeed, phenolic compounds have several health-beneficial effects [19,20]. However, polyphenols have a very poor oral bioavailability due to their low solubility, their sensitivity to gastric pH conditions, and their difficulty to diffuse through lipid cell membranes. Such properties drastically limit their applications in human health. In that context, the use of plant EVs that would naturally contain phenolic compounds could be of great interest to potentially enhance their bioavailability and efficacy [21].

To the best of our knowledge, EVs have not yet been evidenced in olive oil by-products, which are also an abundant source of phenolic compounds. Olive oil by-products (pomaces and wastewaters [WWs]) are naturally rich in phenolic compounds, such as oleuropein (OL), hydroxytyrosol (HT), and tyrosol, with strong antioxidants and radical scavenging properties [22]. In this context, the objective of this work was to screen for the potential presence of EV in these by-products, and then, to isolate and characterize them. To this aim, the presence and nature of EV in olive pomace and WWs, issued from two- and three-phase olive oil mills, respectively, were determined with a focus on the physical properties of vesicles, the class and composition of lipids constituting their lipid membrane, and the nature of the phenolic compounds they contained. Finally, their potential radical scavenging activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Phosphatidylcholine (PC), phosphatidylserine (PS), monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG) were all purchased from Avanti Polar Lipids. 3-Phosphatidic acid (PA), 1-monoolein, and diolein were purchased from Larodan. LipidTOX was provided by Thermo Fischer Scientific. Phosphate buffer solution (PBS), pH 7.2, rhodamine phosphatidylethanolamine (PE), Fast Green, HT ≥98%, verbascoside (VB), ≥99%, and all other phenolic compounds standards, triolein, oleic acid (FFA), PE DPPH, and all solvents were high performance liquid chromatography (HPLC) or analytical grade and were purchased from Sigma Aldrich.

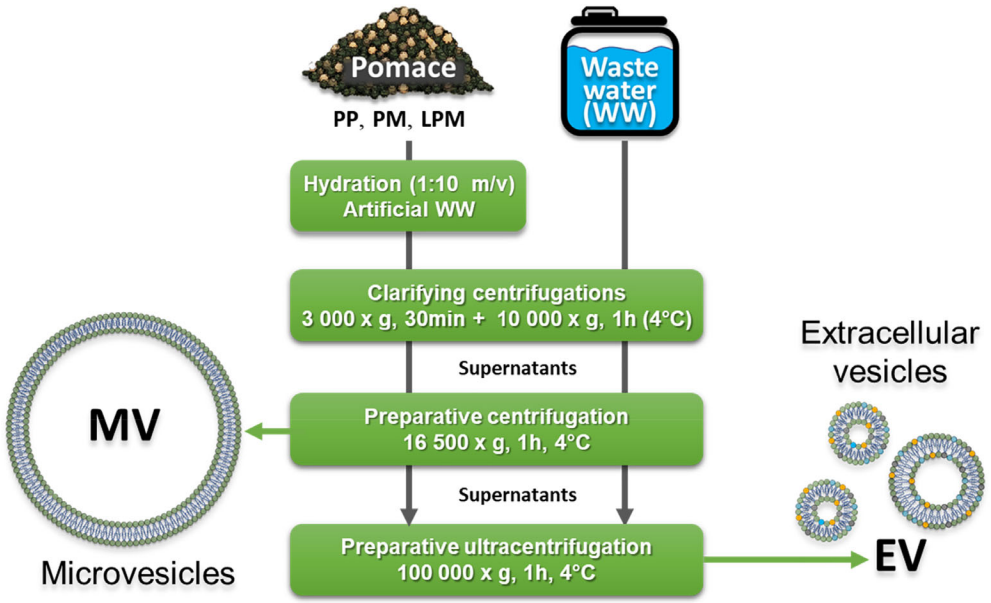

FIGURE 1 Extracellular vesicles and microvesicles isolation from natural and artificial wastewaters. LPM, freeze-dried monovarietal pomace; PM, monovarietal pomace; PP, plurivarietal pomace.

2.2 | Raw materials and sample conditioning

Olive oil by-products (pomace and WW) were collected from three different olive oil mills around Montpellier (France): monovarietal pomaces (MPs) (Picholine) and plurivarietal pomaces (PPs) issued from two different mills and a two-phase extraction process, and WW obtained from a three-phase extraction process.

Prior to their characterization and processing, fresh raw materials were stabilized as follows: MP and PP were oven-dried at 40°C under vacuum (Bioblock Scientific/45001). WW and a part of MP and PP were also freeze-dried at −50°C and 0.2 mbar (Martin Christ Beta/1-8) until water activity was lower than 0.2. Dried pomaces were ground on a Retsch SM300 cutting mill (Retsch GMBH) operated at 2000 rpm with a 2 mm mesh grid. All samples were frozen and kept at −20°C before analytical characterization.

2.3 | Extracellular vesicle and microvesicle isolation

2.3.1 | Production of artificial wastewaters

First, both MP and PP were hydrated separately with ultrapure water in order to reach the average same dry matter content (DM) as in typical olive mill WWs (10% DM). Then, the raw suspension was dispersed for 2 min using high-speed homogenizer IKA T25 ULTRA-TURRAX (IKA) and stirred at 250 rpm for 1 h at 20°C in an orbital shaker (KS 4000i control, IKA). The artificial WW thus obtained was kept at −80°C until being processed.

2.3.2 | Isolation of extracellular vesicles and microvesicles from natural and artificial wastewaters (Figure 1)

Two clarifying centrifugations at 3000 × *g* for 30 min, then 10 000 × *g* for 1 h (Avanti J-C JA 40, Beckman coulter) were performed at 4°C to separate debris, large particles, plant fibers, and intact organelles. Then, a first preparative centrifugation at 16 500 × *g* for 1 h at 4°C (Avanti JXN-26/rotor JA-14.50, Beckman coulter) was done to pellet larger vesicles that will be referred to as microvesicles (MVs). The supernatant was submitted to a subsequent preparative ultracentrifugation at 100 000 × *g* for 1 h at 4°C (Optima L80 XP/rotor 45 Ti, Beckman coulter) to pellet the small nanovesicles that correspond to EVs. EVs obtained after the last centrifugation were collected for further characterization. Pellets of MV obtained after centrifugation at 16 500 × *g* were also recovered for characterization and comparison purposes.

2.4 | Chemical characterizations

2.4.1 | Extraction and content determination of phenolic compounds

From olive oil by-products

Olive oil by-products (WW, MP, and PP) were first defatted with hexane using a Soxhlet apparatus, followed by an air-desolventizing step overnight under fume hood. The extraction of phenolic compounds from defatted samples was carried out following the procedure

described by Dubravka et al. [23] with some modifications: Samples of 50 mg were weighed into a 20 mL brown flask, and 5 mL of ethanol/water (50:50 v/v) were added. Hermetically closed flask was then incubated in an orbital shaker (KS 4000 ic control, IKA) for 60 min at 70°C and 250 rpm, then cooled to room temperature for 5 min, and finally centrifuged for 5 min at 1751 × *g* and 20°C (CR4-12 refrigerated centrifuge, Jouan). The ethanol/water phase (supernatant) was collected and kept at −20°C until analysis. All experiments were done in triplicates.

The extract samples previously obtained were filtered (0.45 µm nylon filters) before HPLC analysis. HPLC analyses were carried out on a Shimadzu LC-20AD solvent delivery unit equipped with a SPD-M20A diode array detector and a column oven CTO-10ASVP (Shimadzu), using a Kinetex C18 reversed-phase column (100 Å, 5 µm, 4.6 × 250 mm^2; Phenomenex). The mobile phase was a mixture of water:acetic acid (99.9:0.1, v/v) (solvent A) and methanol:acetic acid (99.9:0.1, v/v) (solvent B). The following gradient was applied: 0–5 min, isocratic at 15% B; 5–30 min, linear gradient to 80% B; 30–31 min, linear gradient to 100%; 31–35 min, isocratic at 100% B; 35–36 min, linear gradient to 15% B; 36–40 min, equilibration at 15% B. Data were collected at 280 and 330 nm.

For calibration curves, we set from HT (calibration range from 90 to 1600 µM) and VB (calibration range from 25 to 460 µM) methanolic solutions as external standards. The total phenolic content was calculated from the total area of HT and VB according to their respective lambda max, that is, 280 and 330 nm. Individual quantifications were done on HT, OL (HT eq) and VB (VB eq). All experiments were done in triplicates.

Phenolic compounds were identified by UPLC–PDA–ESI/MS. This was performed on an UPLC ACQUITY H-Class (Waters L12QSM439A) and SYNAPT G2-S (Waters, UEB205). The extracts were analyzed using a gradient mixture of water:acetic acid (99.9:0.1, v/v) (solvent A) and methanol:acetic acid (99.9:0.1, v/v) (solvent B). The separation was affected using a linear gradient at 60°C with a flow of 0.2 mL min^{-1} as follows: 15% B at 0–1 min, 15%–80% B at 1–6.5 min, 80%–100% B at 6.5–7 min, 100% isocratic B at 7–8.5 min, 100%–15% B at 8.5–12 min, 15% isocratic B at 12–25 min. The injection volume was 1 µL. For ESI/MS analysis, the positive capillary voltage was set at 3000 V and the negative at 3000 V. The drying gas temperature was 450°C. The samples were analyzed using a full scan from 100 to 1500 *m/z* in positive ionization mode.

From microvesicles and extracellular vesicles

In order to simultaneously extract phenolic compounds and lipids, the Folch extraction procedure was applied on MV and EV, using chloroform:methanol:water (8:4:3, v/v/v) mixture, with a solvent-to-sample ratio of 20:1. Briefly, 80 µL HCl 0.1 N, 1.2 mL Folch mixture, and 100 µL of sample were vortexed for 30 s in a test tube closed with a screw cap. Then, 300 µL of Folch mixture and 25 µL NaCl 0.73% were added to the tube and vortexed again for 30 s. After centrifugation of the tubes for 5 min at 778 × *g*, chloroform and ethanolic phases were recovered separately. The non-polar (chloroform) phase was dedicated to lipid analysis, and the polar one to phenolic compounds analysis by HPLC according to the procedure described in Section 2.4.1.1.

2.4.2 | TLC-densitometry on lipid extracts from by-products, microvesicles, and extracellular vesicles

The lipid extract solutions previously obtained were analyzed without dilution according to the following procedure: TLC chromatography was carried out on HPTLC silica gel 60 pre-coated plates (Merck). Lipid extracts and standard solutions were sprayed on 3 mm width bands, using a CAMAG ATS4 apparatus.

In order to visualize all lipid compounds of interest (polar lipids [phospholipids and mono and digalactosyl lipids] as well as non-polar lipids [triacylglycerols, partial acylglycerols, and free fatty acids]), a two-step development was performed on HPTLC silica gel 60 precoated plates as follows:

- First step: 40 mm with $CHCl_3$/MeOH/H_2O (19:4:0.5 v/v/v)
- Second step: 80 mm with hexane/diethyl ether/formic acid (14:6:0.2 v/v/v)

Plates were then dipped in aqueous copper sulfate:phosphoric acid 85%:ethanol:water (50:40:25:390 v/v/v/v) solution, then dried and heated for 8 min at 150°C. The plates were scanned using CAMAG TLC scanner3 at 500 nm.

Compounds were identified by comparing their frontal ratio to authentic standards. Quantification was made using standard calibration curves.

2.4.3 | Fatty acid composition by GC on lipid extracts from by-products, microvesicles, and extracellular vesicles

Fatty acid methyl esters (FAMEs) aliquots (5 µL) of Folch extracts were analyzed by GC (Focus GC, Thermo Electron Corporation) equipped with a split injector (ratio of 1/20), a CP-Cil 88 Varian capillary column (50 m × 0.25 mm with a 0.2 µm thick film; Chrompack) and 1 mL min^{-1} of helium as carrier gas. FAMEs were analyzed using a flame ionization detector and ChromCard Data System (version 2005; Thermo Electron Corporation).

The column temperature started at 150°C, increased from 150 to 225°C at a rate of 5°C min^{-1} and maintained at 225°C for 10 min. The injector and detector temperatures were 250 and 270°C, respectively. FAMEs were identified using a mixture of methyl esters as external standard. Each sample was analyzed with three repetitions.

2.5 | Physical characterization of extracellular vesicles

A Nicomp dynamic light scattering (DLS) Nanoparticle Size Analyzer (Nicomp, Nano Z3000) was used for characterizing the particle size and charge in EV. In this aim, a given volume of EV was first diluted 200 times in PBS (pH 7.2). Then, an aliquot was further diluted 10 times in the same buffer and filtered on 0.2 µm filters. The filtrated dispersion of 2.5 mL was inserted in 3 mL quartz cube for DLS measurement.

For pH measurement by electrophoretic mobility, a similarly prepared sample was placed in a clear disposable zeta cell, and zeta potential was measured at different pH values (2–7.2) after adjustment with HCl 0.1 N. Measurements were made under the following parameters: viscosity on 0.891; liquid index at 1331 and E. Field set on 1 V cm^{-1} for zeta potential.

Laser confocal scanning microscopy (Confocal Leica SP8) was then used to determine the structure of the different assemblies in EV. Three different fluorescent probes were used for sample labeling: rhodamine PE for polar lipids, LipidTOX for non-polar lipids, and Fast Green for proteins. Images were taken on 10 µL of the preparation in both transmission and reflection modes. The different sequences (1–3) representing non-polar lipids (green), polar lipids (red), and proteins (blue) were visualized using the following laser wavelengths: 488, 561, and 633 nm, respectively.

Transmission electron cryomicroscopy (CryoTEM) observations were conducted to get a finer description of the EV structure. For this analysis, fresh isolated EVs were first diluted 10 times in ultrapure water and filtrated at 0.45 µm. EV suspensions (3 µL) were then placed to glow-discharged Lacey grids (Ted Pella), blotted for 1 s and then flash frozen in liquid ethane using CP3 Cryoplunge 3 system with GentleBlot technology (Gatan) where temperature was maintained at −172°C. Cryo-EM observation was carried out on a JEOL 2200FS Field Emission Gun Electron Microscope (JEOL (EUROPE) SAS) operating at 200 kV under low-dose conditions (total dose of 20 electrons/$Å^2$) in the zero-energy-loss mode with a slit width of 20 eV. Images were taken with a 4k × 4k slow-scan CCD camera (Gatan) with a defocus ranging from 1.4 to 1.8 µm.

2.6 | DPPH assay of extracellular vesicle extracts in comparison with their original by-products

The radical scavenging activity of EV and by-product ethanolic extracts was evaluated with the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [24]. Briefly, a methanol fresh solution of DPPH radical (180 µL at 50 µM) was mixed with 20 µL of the tested ethanolic extract solutions at various concentrations: Four different concentrations were tested each time, with 4, 12, 24, and 48 µL of initial ethanolic solutions that were diluted in 2 mL of ethanol. Ethanol was used as blank reference and HT and Trolox as reference antioxidants (used at 1, 10, and 20 µM concentrations). The mixture was incubated in the dark at 25°C and absorbance was measured at 515 nm from 0 to 480 min on a Tecan Infinite M1000 PRO Microplate Reader (Tecan). Results were expressed as EC50, which corresponds to the amount of compound able to reduce 50% of the initial DPPH.

2.7 | Statistical analysis

ANOVA analyses were performed using the statistics software R (version 3.5-1). Means and standard deviations were performed using Microsoft Excel 2016 software. The significance level α of statistical analysis was set to 0.05.

3 | RESULTS AND DISCUSSION

3.1 | Dry matter of olive oil by-products

First, the global compositions of by-products and isolated MV and EV were evaluated (Table 1). DM accounted for 40.3 (1.8) % in MP, 38.2 (0.9) % in PP, and 13.1 (0.2) % in WW. It is worth noting that the DM of the used WW was relatively high in comparison with other reported values [25]. The significant difference in DM concentration between PP and WW can be attributed to the water quantity supplement in the three-phase olive mill process. Indeed, it is known that the typical water content in olive pomaces depends on the extraction process with around 25%–30%, 45%, and 55%–70%, respectively, in pomaces from the traditional extraction system, from the three-phase extraction, and from the two-phase extraction systems [26]. DM represented 1.6%–4.6% for MV and EV, which means that an important amount of olive by-product constituents were lost during their recovery in ultrapure water after several centrifugation steps that can lead to the loss of solid fractions of interest.

3.2 | Lipid content and profile of olive oil by-products

Lipid content (% DM) was found to be higher in WW (24.1 (0.9) %) than in MPs (10.6 (0.3) %) or PPs (10.7 (0.7) %). Lipid content in olive by-products depends on several factors, mainly on the initial oil content in the olive fruit. This content tends in particular to increase significantly at the end of the harvest period [27]. Lipid content in EV fractions dropped drastically with 3.4 (0.2) (% DM) for WWEV, 7.7 (0.3) % and 5.9 (0.9) % for EV, respectively, from PPs (PPEV) or MPs (MPEVs).

Lipid classes were then analyzed in all different samples by TLC with a specific focus on the main membrane lipids, namely, phospholipids (PC, PA, or PE) or galactolipids (MGDG or DGDG) (Figure 2). It is important to note that PC/PA and DGDG/PE are co-eluted and were evaluated using, respectively, PC and DGDG calibration curves.

A detailed quantification of these polar lipids was carried out for each fraction (Figure 3). For co-products, WW were richer in polar lipids than pomaces, either mono- or plurivarietal (3.8% vs. 0.5% or 0.6%). For EVs, polar lipids represented around 49% of total lipids, and their profiles were globally similar in all EVs. PC/PA

TABLE 1 Global composition of raw materials (wastewaters, plurivarietal, and monovarietal pomaces), microvesicles and extracellular vesicles (dry matter, lipid content, and phenolic compounds) (Triplicate ± stand for standard deviation).

	Dry extract (% WM)	Lipid content (% DM)	Phenolic compounds (mg HT eq g^{-1} of raw material)	HT (mg g^{-1}) (λ_{max} 280 nm)	OL (mg HT eq g^{-1}) (λ_{max} 280 nm)	VB (mg g^{-1}) (λ_{max} 330 nm)
WW	13.1 (0.2)^{b}	24.1 (0.9)^{b}	55.6 (2.9)^{a}	0.5 (0.7)^{ab}	2.4 (1.0)^{a}	1.7 (0.5)^{a}
PP	38.2 (0.9)^{a}	10.7 (0.7)^{d}	22.9 (0.1)^{b}	0.4 (0.1)^{b}	0.3 (0.0)^{a}	0.2 (0.3)^{b}
MP	40.3 (1.8)^{a}	10.6 (0.3)^{d}	12.4 (0.2)^{c}	1.3 (0.3)^{a}	2.2 (0.9)^{a}	0.0 (0.0)^{b}
WWMV	2.6 (0.5)^{cde}	19.3 (1.4)^{c}	4.6 (3.1)^{d}	0.2 (0.2)^{b}	0.1 (0.2)^{a}	0.1 (0.1)^{b}
PPMV	3.1 (0.3)^{cde}	31.9 (1.9)^{a}	3.2 (2.4)^{d}	0.1 (0.1)^{b}	1.8 (1.9)^{a}	0.2 (0.1)^{b}
MPMV	4.4 (0.8)^{cd}	19.6 (0.7)^{c}	5.2 (0.8)^{d}	0.2 (0.1)^{b}	1.6 (1.5)^{a}	0.3 (0.1)^{b}
LMPMV	1.7 (0.5)^{e}	17.5 (0.8)^{c}	3.1 (1.1)^{d}	0.1 (0.1)^{b}	0.4 (0.7)^{a}	0.3 (0.1)^{b}
WWEV	4.6 (0.4)^{c}	3.4 (0.2)^{f}	3.9 (0.8)^{d}	0.1 (0.0)^{b}	0.1 (0.0)^{a}	0.1 (0.0)^{b}
PPEV	2.4 (0.4)^{de}	7.7 (0.3)^{de}	2.1 (0.3)^{d}	#	<0.1	#
MPEV	1.6 (0.3)^{e}	5.9 (0.9)^{ef}	4.6 (0.6)^{d}	#	<0.1	#
LMPEV	2.3 (0.8)^{de}	6.7 (0.7)^{ef}	2.7 (0.0)^{d}	#	<0.1	#

Note: Different superscript letters within a same column stand for significant difference using ANOVA with $p < 0.05$. "#" stands for not detected.
Abbreviations: DM, dry matter; HT, hydroxytyrosol; LMPEVs, freeze-dried monovarietal pomace extracellular vesicles; LMPMVs, freeze-dried monovarietal pomace microvesicles; MPEVs, monovarietal pomace extracellular vesicles; MPMVs, monovarietal pomace microvesicles; OL, oleuropein; PPEVs, plurivarietal pomace extracellular vesicles; PPMVs, plurivarietal pomace microvesicles; VB, verbascoside; WM, wet matter; WWEVs, wastewater extracellular vesicles; WWMVs, wastewater microvesicles.

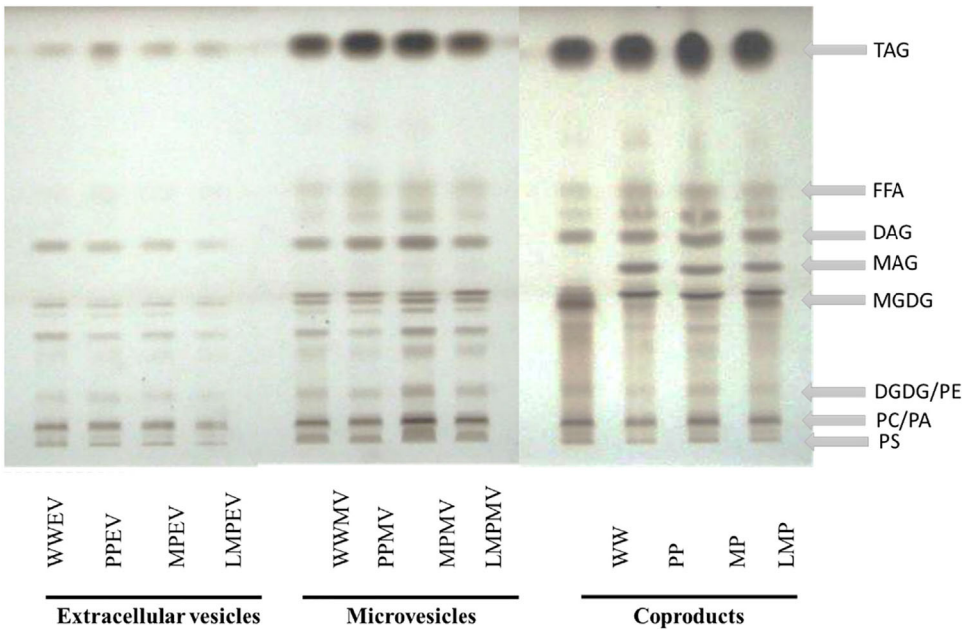

FIGURE 2 Representative HPTLC obtained for extracellular vesicles (EVs), microvesicles (MVs), and by-products (analysis development: first step—40 mm with $CHCl_3$/MeOH/H_2O [19:4:0.5 v/v/v] and second step—80 mm with hexane/diethyl ether/formic acid [14:6:0.2 v/v/v]). After the plate was dipped in aqueous copper sulfate:phosphoric acid 85%:ethanol:water (50:40:25:390 v/v/v/v) solution, it was dried and heated for 8 min at 150°C. Main lipids were identified in comparison with commercially available standards. LMP, freeze-dried monovarietal pomace; LMPEVs, freeze-dried monovarietal pomace extracellular vesicles; LMPMVs, freeze-dried monovarietal pomace microvesicles; MP, monovarietal pomace; MPEVs, monovarietal pomace extracellular vesicles; MPMVs, monovarietal pomace microvesicles; PP, plurivarietal pomace; PPEVs, plurivarietal pomace extracellular vesicles; PPMVs, plurivarietal pomace microvesicles; WW, wastewater; WWEVs, wastewater extracellular vesicles; WWMVs, wastewater microvesicles.

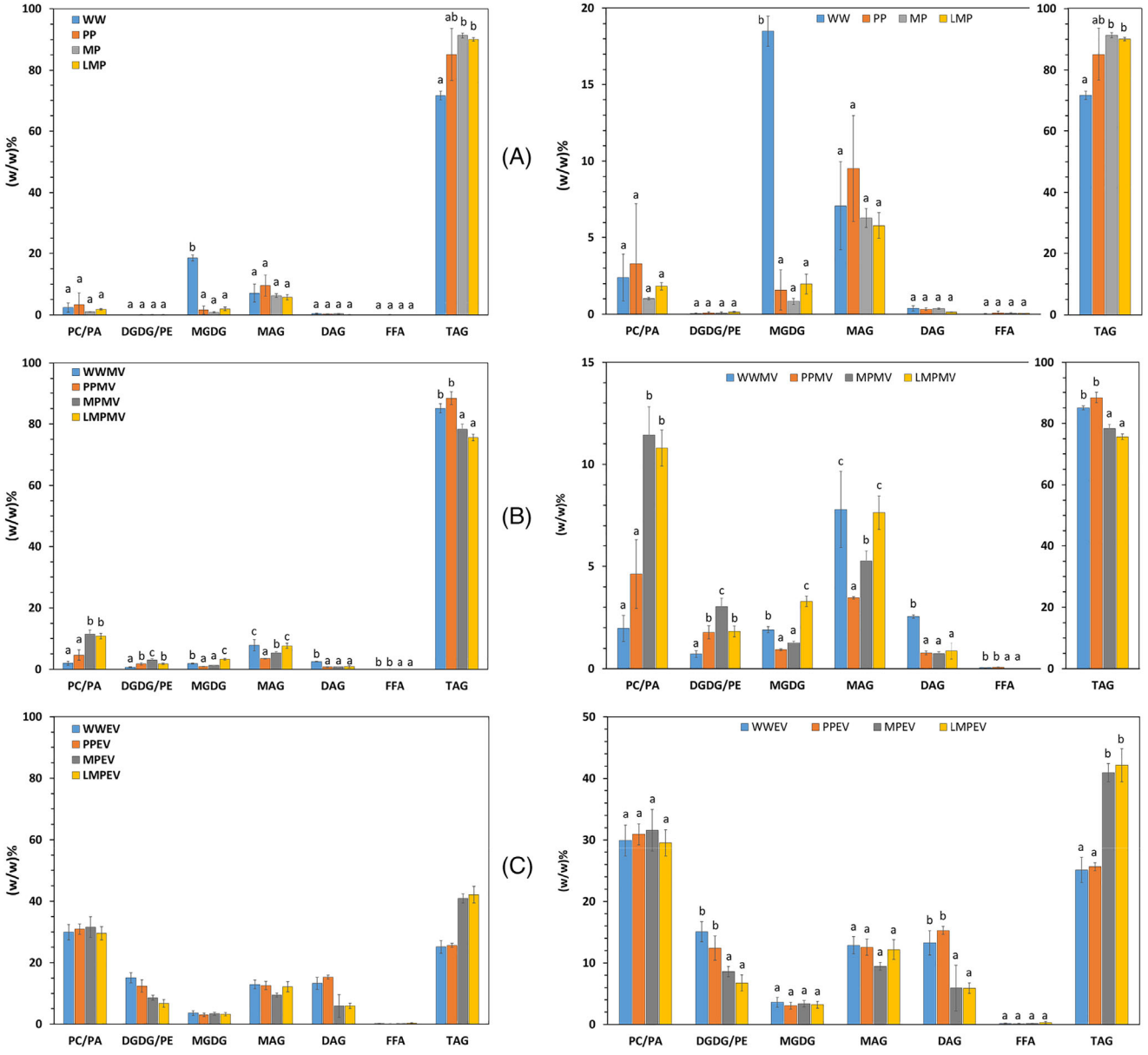

FIGURE 3 Quantification of the main lipids by thin layer chromatography in (A) by-products, (B) microvesicles, and (C) extracellular vesicles. LMP, freeze-dried monovarietal pomace; LMPEVs: freeze-dried monovarietal pomace extracellular vesicles; LMPMVs, freeze-dried monovarietal pomace microvesicles; MP, monovarietal pomace; MPEVs, monovarietal pomace extracellular vesicles; MPMVs, monovarietal pomace microvesicles; PP, plurivarietal pomace; PPEVs, plurivarietal pomace extracellular vesicles; PPMVs, plurivarietal pomace microvesicles; WW, wastewater; WWEVs, wastewater extracellular vesicles; WWMVs, wastewater microvesicles.

were the more abundant molecules followed by DGDG/PE. Accordingly, EV selectively concentrates polar lipids in comparison with their native by-products (≃10 times) or their corresponding MV (3–12 times).

This observation can be explained by the structure of biological membranes, which are composed mainly of polar lipids [10,28]. Indeed, plant plasma membrane and plant chloroplast membrane have quite specific and well-conserved compositions. Plant plasma membrane are mainly made of PC and PE (68%–80% wt.), followed by phosphatidylglycerol, PI, PS, and PA. Thylakoids are made up of neutral galactolipids in the form of MGDG (53% wt.) and DGDG (27% wt.) [29]. Accordingly, the polar lipid composition found in the EV fractions could result from a blend of plasma and thylakoid membrane fragments.

These results agree with other studies that found that PC was an important constituent in different vegetal exosome-like nanoparticles, representing 30% in grapefruit [30]. PA dominated in ginger with 40%, and equilibrated amounts were shown recently in *Arabidopsis* leaves [30–32]. PC is an important lipid that contributes to the formation of the membrane bilayer of vesicles [10]. Regarding phosphatidic acid, it is an important lipid mediator controlling membrane fusion and fission, its role is important in vesicular formation. In literature, lipid analysis of EV from plant origin has often shown relatively different profiles depend-

ing on the source. These variations can be attributed to the organelle origins of the vesicle [13].

The fatty acid composition of by-products and vesicle fractions was globally comparable, with FFA being the most represented (>60%) (Table 2). However, with the exception of PP microvesicles, MV and EV seem to be selectively concentrated in alpha-linolenic acid (C18:3, ALA), especially for EV. This increase in ALA can be explained by their relative enrichment in galactolipids that are constituents of all types of membranes, in particular EV structures in plants, and are very rich in ALA [33].

As shown by TLC analysis, non-polar lipids were also present in the vesicle fractions, with triacylglycerols being the most abundant followed by diacylglycerols and monoacylglycerols. The presence of such compounds, which are non-membranous lipids, could be attributed to contamination of the vesicular fraction. This problem is generally due to the purification technique. Here, differential ultracentrifugation was used, the efficiency of which depends on several factors, such as density, size, viscosity, and the material used. For example, differential ultracentrifugation is sometimes non-efficient in separating very heterogeneous populations and vesicle subtypes [34]. In some other studies, various techniques were combined with differential centrifugation, including filtration or size exclusion chromatography leading to better vesicle purification and the elimination of non-vesicular material [35–37].

3.3 | Phenolic content and profile of olive oil by-products

Total phenolic contents (expressed as HT equivalents) were evaluated in WW, MP, and PPs and their resulting MV or EV (Table 1). WW was found to be the most concentrated in phenolic compounds (55.6 (2.9) mg g^{-1}) followed by PPs (22.9 (0.1) mg g^{-1}) and MPs (12.4 (0.2) mg g^{-1}). The higher concentration of phenolic compounds in WW compared to pomaces is generally attributed to the polarities of such molecules, which tend to locate more easily in a strong aqueous environment such as WW. These results have already been discussed by others, who have also shown that the content in phenolic compounds is dependent on the extraction process used and the type of by-products that are generated [25,38]. Phenolic compounds were much less concentrated in MV fractions, ranging from 3.1 (0.3) (freeze-dried MP microvesicles) to 5.2 (0.8) mg g^{-1} HT eq g^{-1} raw material (MP microvesicles). This effect was even more pronounced in EV fractions from 2.1 (0.3) for PPEV to 4.6 (0.6) for MPEV.

A more detailed phenolic profile of by-products and vesicle fractions was performed by HPLC and HPLC–MS (Figure 4). In both WW or MPs and PPs, six different phenolic compounds represented around 60% of total peak area. These six molecules were identified as HT (m/z = 153.1), βhydroxyverbascoside (m/z = 639.4), VB (m/z = 623.1), kaempferol-7-o-glucoside (m/z = 447.2), OL (m/z = 539.5), and an unidentified compound with an m/z = 535.2. Some authors tentatively identified this compound as 6′-rhamnopyranosyl oleoside [39,40]. However, due to the fact that this unknown compound is visible when

TABLE 2 Total fatty acid profiles of by-products and vesicular fractions ("Triplicate" stands for standard deviation).

Fatty acid	WW	PP	MP	WWMV	PPMV	MPMV	LMPMV	WWEV	PPEV	MPEV	LPMEV
C16:0	10.3 (0.0)^{G}	13.3 (0)^{E}	12.2 (0.1)^{D}	10.5 (0.1)^{G}	13.4 (0.1)^{D}	13.5 (0.3)^{D}	11.9 (0.2)^{F}	11.9 (0.0)^{EF}	16.2 (0.1)^{A}	15.0 (0.1)^{B}	14.1 (0.2)^{C}
C16:1	0.6 (0.0) ^{ABC}	0.8 (0.1)^{AB}	0.7 (0.1)^{A}	0.6 (0.1)^{ABC}	0.9 (0.1)^{A}	0.8 (0.0)^{AB}	0.6 (0.1)^{C}	0.4 (0.0)^{BC}	0.8 (0.1)^{AB}	0.5 (0.0)^{ABC}	0.4 (0.5)^{C}
C18:0	3.0 (0.1)^{AB}	2.9 (0.0)^{B}	1.4 (1.8)^{AB}	3.3 (0.0)^{A}	3.2 (0.0)^{A}	3.0 (0.0)^{AB}	3.0 (0.0)^{AB}	2.8 (0.0)^{AB}	3.0 (0.1)^{AB}	3.1 (0.0)^{AB}	2.9 (0.0)^{AB}
C18:1	73.2 (0.1)^{A}	68.4 (0.2)^{E}	66.5 (0.1)^{D}	72.0 (0.1)^{B}	69.2 (0.3)^{C}	64.7 (0.0)^{G}	65.6 (0.1)^{F}	65.8 (0.7)^{F}	59.4 (0.1)^{I}	60.0 (0.1)^{I}	60.7 (0.7)^{H}
C18:2 (n-6)	10.1 (0.1)^{H}	11.8 (0.0)^{C}	15.2 (0.1)^{F}	10.2 (0.2)^{H}	10.9 (0.0)^{G}	14.6 (0.0)^{D}	15.3 (0.3)^{BC}	12.3 (0.1)^{E}	14.7 (0.1)^{D}	15.6 (0.1)^{AB}	16.0 (0.3)^{A}
C20:0	0.4 (0.0)^{CD}	0.4 (0.0)^{DEF}	0.4 (0.0)^{CDE}	0.4 (0.0)^{EF}	0.4 (0.0)^{CDEF}	0.4 (0.0)^{EF}	0.4 (0.0)^{F}	0.5 (0.0)^{A}	0.5 (0.0)^{B}	0.4 (0.0)^{BC}	0.4 (0.0)^{BCD}
C18:3 (n-3)	1.6 (0.1)^{F}	1.1 (0.0)^{FG}	1.4 (0.1)^{G}	1.9 (0.0)^{E}	1.3 (0.0)^{FG}	2.3 (0.0)^{DE}	2.4 (0.1)^{FD}	4.7 (0.3)^{A}	3.6 (0.0)^{C}	4.2 (0.3)^{B}	4.3 (0.0)^{B}
C22:0	0.1 (0.1)^{F}	0.2 (0.0)^{EF}	0.3 (0.1)^{DE}	0.2 (0.0)^{EF}	0.2 (0.0)^{EF}	0.1 (0.0)^{EF}	0.2 (0.0)^{DE}	0.3 (0.1)^{AB}	0.4 (0.0)^{A}	0.3 (0.1)^{CD}	0.3 (0.0)^{BC}
C20:4 (n-6)	0.3 (0.0)^{A}	0.2 (0.0)^{A}	0.3 (0.1)^{ABC}	0.2 (0.0)^{BC}	0.1 (0.0)^{BC}	0.2 (0.0)^{AB}	0.2 (0.0)^{AB}	0.1 (0.1)^{C}	0.1 (0.0)^{BC}	0.1 (0.0)^{BC}	0.2 (0.1)^{BC}
Ratio ω6/ω3	6.7 (0.1)^{C}	10.8 (0.0)^{A}	10.9 (0.3)^{A}	5.3 (0.1)^{D}	8.7 (0.1)^{B}	6.5 (0.0)^{C}	6.3 (0.1)^{C}	2.6 (0.2)^{G}	4.2 (0.0)^{E}	3.7 ± 0.2)^{F}	3.7 (0.1)^{F}

Note: Different superscript letters within a same line stand for significant difference using ANOVA with $p < 0.05$.

Abbreviations: LMP, freeze-dried monovarietal pomace; LMPEVs, freeze-dried monovarietal pomace extracellular vesicles; LMPMVs, freeze-dried monovarietal pomace microvesicles; MP, monovarietal pomace; MPEVs, monovarietal pomace extracellular vesicles; MPMVs, monovarietal pomace microvesicles; PP, plurivarietal pomace; PPEVs, plurivarietal pomace extracellular vesicles; PPMVs, plurivarietal pomace microvesicles; WW, wastewater; WWEVs, wastewater extracellular vesicles; WWMVs, wastewater microvesicles.

TABLE 3 Selective concentration of by-products, microvesicles, and extracellular vesicles in dry matter, total lipids, phenolic compounds, and polar lipids.

			Polar lipids				Phenolic compounds			Total lipids	
		Dry matter (%)	On phenolic compounds (%)	On total lipids (%)	On dry matter (%)	On liquid (%)	On total lipids (%)	On dry matter (%)	On liquid (%)	On dry matter (%)	On liquid (%)
Raw materials	**WW**	13.0 (0.2)^{gh}	16.4 (0.6)^{bc}	3.8 (0.2)^{b}	0.9 (0.0)^{ab}	0.1 (0.0)^{ef}	23.2 (1.8)^{g}	5.1 (0.2)^{g}	0.84 (0.04)^{ef}	24.1 (0.9)^{g}	3.6 (0.2)^{fg}
	PP	61.8 (1.2)^{hi}	2.4 (0.4)^{a}	0.5 (0.1)^{a}	0.1 (0.0)^{a}	0.0 (0.0)^{ab}	21.5 (1.5)^{g}	1.2 (0.0)^{efg}	1.42 (0.03)^{f}	10.7 (0.7)^{d}	6.6 (0.5)^{gh}
	MP	67.5 (1.9)^{i}	5.2 (0.8)^{ab}	0.6 (0.1)^{a}	0.1 (0.0)^{a}	0.0 (0.0)^{b}	11.7 (0.3)^{f}	2.2 (0.0)^{fg}	0.84 (0.02)^{ef}	10.6 (0.3)^{d}	7.2 (0.4)^{h}
Microvesicles	**WWMV**	2.7 (0.4)^{cd}	199.5 (75.5)^{cd}	3.7 (0.1)^{b}	0.7 (0.0)^{ab}	0.0 (0.0)^{a}	2.4 (1.2)^{bc}	0.5 (0.2)^{bcd}	0.01 (0.01)^{bcd}	19.3 (1.4)^{ef}	0.5 (0.0)^{cd}
	PPMV	3.3 (0.2)^{de}	936.6 (205.7)^{fg}	8.1 (2.0)^{c}	2.6 (0.8)^{cd}	0.1 (0.0)^{d}	1.0 (0.5)^{a}	0.3 (0.2)^{def}	0.01 (0.01)^{abc}	31.9 (1.9)^{g}	1.0 (0.1)^{ef}
	MPMV	4.7 (0.6)^{ef}	607.8 (97.4)^{e}	16 (1.8)^{d}	3.1 (0.4)^{cd}	0.1 (0.0)^{f}	2.7 (0.4)^{bc}	0.5 (0.1)^{a}	0.02 (0.00)^{de}	19.6 (0.7)^{f}	0.9 (0.1)^{de}
	LMPMV	1.7 (0.3)^{ab}	855.5 (192.4)^{ef}	14.1 (0.6)^{cd}	2.5 (0.0)^{bc}	0.0 (0.0)^{b}	1.8 (0.6)^{ab}	0.3 (0.1)^{abc}	0.01 (0.00)^{a}	17.5 (0.8)^{e}	0.3 (0.0)^{c}
Extracellular vesicles	**WWEV**	4.9 (0.3)^{fg}	388.4 (50.4)^{d}	43.5 (3.8)^{e}	1.5 (0.2)^{cd}	0.1 (0.0)^{cd}	11.5 (1.9)^{f}	0.4 (0.1)^{bcd}	0.02 (0.00)^{cd}	3.4 (0.2)^{a}	0.2 (0.0)^{b}
	PPEV	2.5 (0.3)^{bc}	1709.2 (127.8)^{h}	47.1 (2.9)^{ef}	3.6 (0.2)^{d}	0.1 (0.0)^{d}	2.8 (0.1)^{cd}	0.2 (0.0)^{a}	0.01 (0.00)^{a}	7.7 (0.3)^{c}	0.2 (0.0)^{b}
	MPEV	1.6 (0.2)^{a}	709.0 (127.7)^{ef}	54.9 (5.9)^{g}	3.2 (0.3)^{d}	0.1 (0.0)^{bc}	8.1 (1.8)^{ef}	0.5 (0.0)^{a}	0.01 (0.00)^{abc}	5.9 (0.9)^{b}	0.1 (0.0)^{a}
	LMPEV	2.4 (0.7)^{abc}	1269.8 (190.6)^{gh}	51.0 (1.8)^{fg}	3.4 (0.5)^{cd}	0.1 (0.0)^{cd}	4.1 (0.4)^{de}	0.3 (0.0)^{ab}	0.01 (0.00)^{ab}	6.7 (0.7)^{bc}	0.2 (0.0)^{b}

Note: Triplicate ± stand for standard deviation. Different superscript letters within a same column stand for significant difference using ANOVA with $p < 0.05$.

Abbreviations: LMPEV, freeze-dried monovarietal pomace extracellular vesicle; LMPMV, freeze-dried monovarietal pomace microvesicle; MP, monovarietal pomace; MPEV, monovarietal pomace extracellular vesicle; MPMV, monovarietal pomace microvesicle; PP, plurivarietal pomace; PPEV, plurivarietal pomace extracellular vesicle; PPMV, plurivarietal pomace microvesicle; WW, wastewater; WWEV, wastewater extracellular vesicle; WWMV, wastewater microvesicle.

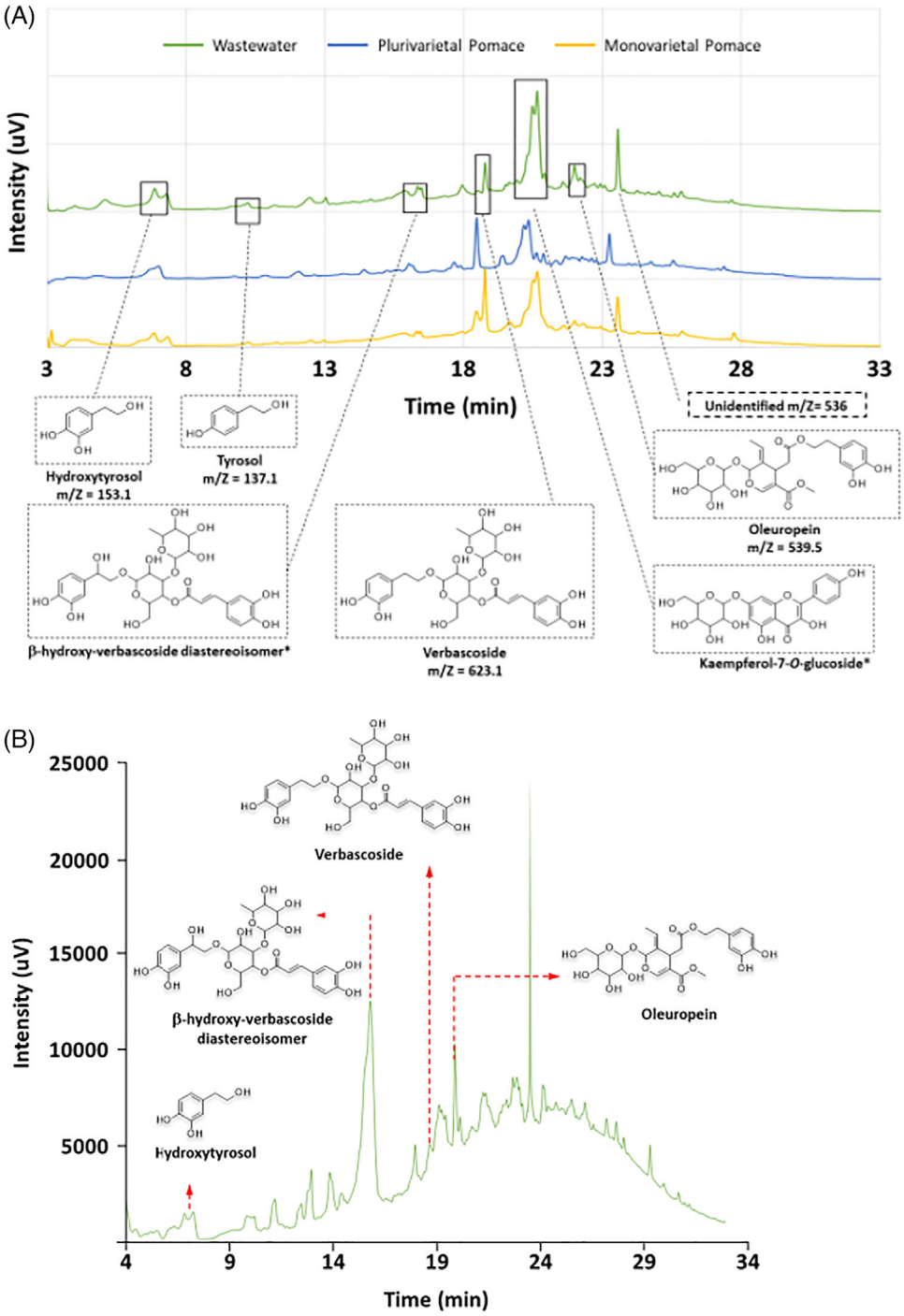

FIGURE 4 HPLC chromatograms obtained at 280 nm for the phenolic compound composition of (A) by-products (blue: monovarial pomace, orange: plurivarietal pomace, grey: wastewater), and an example obtained for (B) wastewater extracellular vesicles (WWEVs).

analyzed at 280 nm and its high retention time than OL, we consider that it could better correspond to *p*-coumaroyl-6′-secologanoside as suggested by others [41–43]. Overall, these five different phenolic compounds represented around 60% of total peak area. The free main quantified compounds are HT, OL, and VB, which also correspond to those described in the literature and whose levels vary from one study to another. This is always explained by environmental or cultivar factors [27]. We found that WW and MP contain higher contents of OL than other phenolic compounds, namely, HT and VB; these results are in agreement with literature [44–46]. OL is a molecule made up of three subunits: HT (4-(2-hydroxyethyl)benzene-1,2-diol), oleanolic acid, and glucose. Storage of the fruit before oil extraction as well as the trituration process promote enzymatic activities, resulting in the hydrolysis of OL and the release of HT [47].

Phenolic compounds were also found in MV and EV but with a much lower concentration than in initial by-products (Table 1). Among the dif-

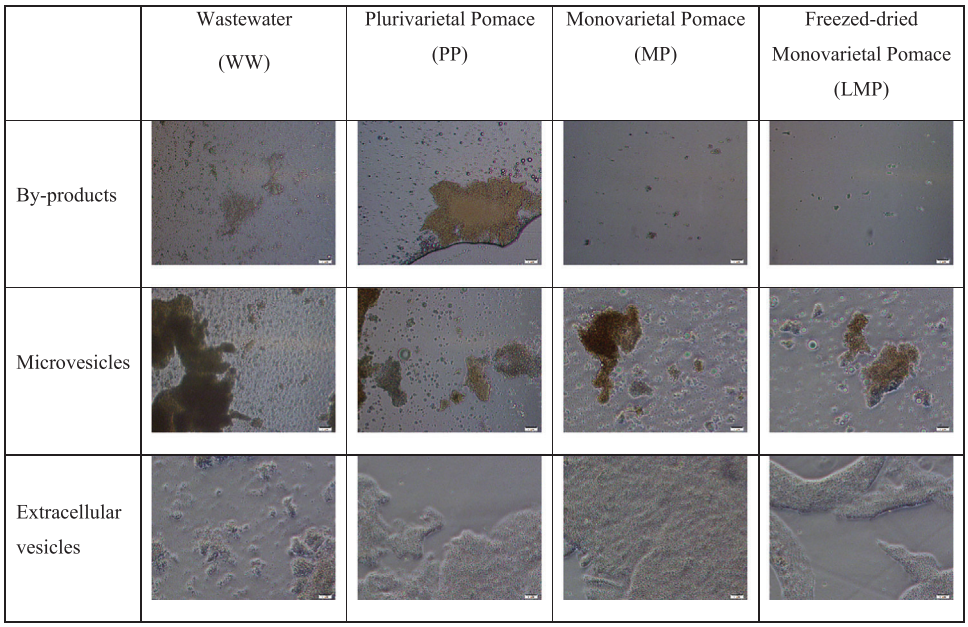

FIGURE 5 Optical microscopy of by-products, microvesicles, and extracellular vesicles.

ferent EVs, those originating from WW were the most concentrated in phenolics. MV contained the three phenolic compounds identified previously in the by-products, namely, HT, OL, and VB. OL and VB were also identified in all final exosomal fractions, unlike HT, which was only detected in EV from WW. This can be attributed to the high initial abundance of phenolic compounds in WW. It is worth noting that the relative profile in phenolic compounds was specific in the vesicular fractions. However, in all vesicular fractions, this profile was dominated by the unidentified compounds with *m/z* 536.2, which was already observed by others for olive extracts [40–42]. Finally, results have shown that freeze-drying affects the amount of phenolic compounds negatively [48], which can be explained by the disruption of fruit cells, leading to the activation of certain enzymes contributing to the degradation of the compounds [49], or the release of phenolic compounds outside the matrix [50].

3.4 | Relative contents of total lipids, polar lipids, and phenolic compounds

Concentrations in total lipids, polar lipids, and phenolic compounds were finally expressed out of wet matter or DM or out of total lipid or phenolic compounds in original by-products or in vesicular fractions (Table 3). Such ratios help visualizing the selective concentration in some of the compounds in the isolated vesicular fractions and, more specifically, in EV compared to the original by-products. We already underlined that MVs are (on DM) more concentrated in lipids (two to three times) than their corresponding native by-products with the exception of MV from WW (Table 1). Conversely, phenolic compounds are more concentrated in by-products (WWs and pomaces) than in MV or EV fractions. This loss in phenolic compounds results in lower phenolic compounds/DM and phenolic compounds/total lipid ratios than the ones of initial by-products. It is also worth noting the polar lipids are more concentrated out of DM and total lipids except for WWMV and out of phenolic compounds for all MV. For EV, and based on phenolic compounds or total lipids, polar lipids appear to be more concentrated than their corresponding by-products or MV. On the opposite, phenolic compounds and total lipids are less concentrated on the same basis in EV than in by-products or MV.

3.5 | Size, charge, and structure of by-products or vesicular fractions

The global physical structures of the different fractions (by-products, MV, and EV) were observed by optical microscopy (Figure 5). Among by-products, WW and PP displayed zones of aggregated lipids. Following centrifugations, this aggregation was reinforced and observed in all MV images. On the contrary, final extracellular fractions, although obtained after 100 000 *g* ultracentrifugation, appeared homogeneous.

The microstructure of the different samples was further investigated by CLSM (Figure 6A) using three complementary types of probes: rhodamine PE for polar lipids, LipidTOX for apolar lipids, and Fast Green for proteins. For all fractions, all these constituents were assembled as spherical forms for lipids and polar lipids, whereas proteins were more commonly found as aggregates. These aggregates seemed more concentrated in EV from WW and PPs. Cryo-TEM (Figure 6B) on EV-filtrated samples (0.45 μm) evidenced the presence of small and large unilamellar vesicles in the fractions with scales that ranged, respectively, from 50 to 200 nm for small ones and 100–1000 nm for larger ones. The fact that CryoTEM analysis shows the presence of spherical vesicles, delimited by a single bilayer of amphiphilic

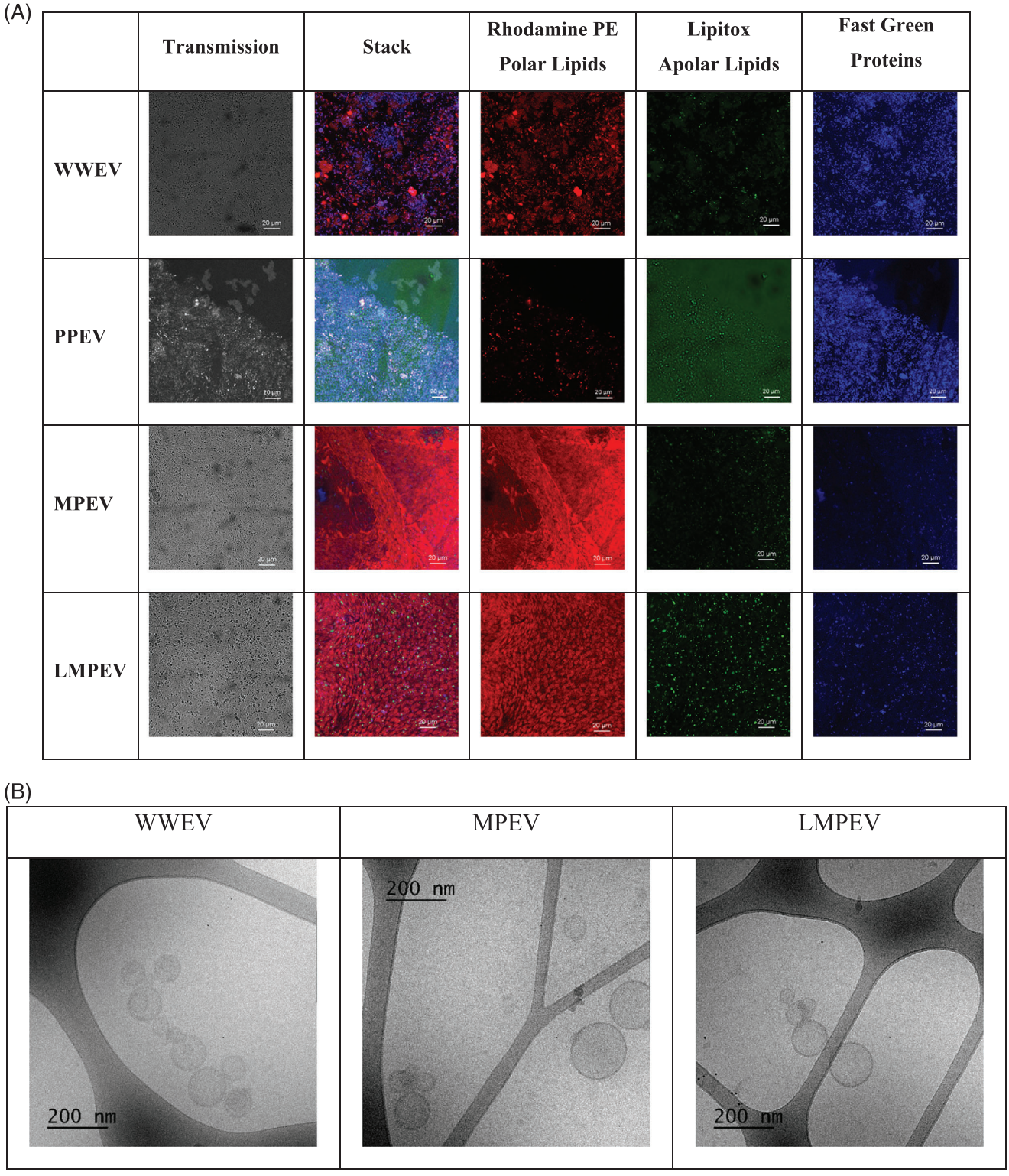

FIGURE 6 Confocal microscopy (red dye: PL, blue dye: proteins, green dye: triolein [TAG]) (A) and cryoTEM (B) of extracellular vesicles: freeze-dried monovarietal pomace extracellular vesicles (LMPEV), monovarietal pomace extracellular vesicles (MPEV), plurivarietal pomace extracellular vesicles (PPEV), and wastewater extracellular vesicles (WWEV).

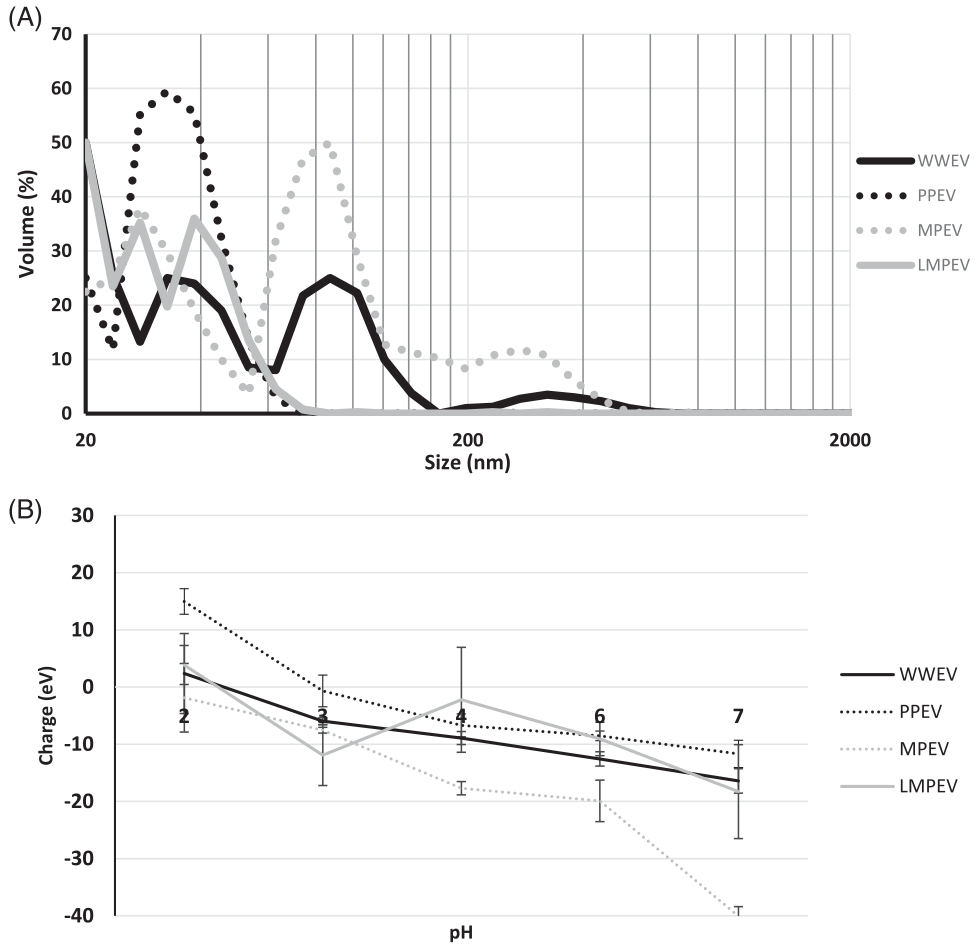

FIGURE 7 Droplet size distributions (A) and zeta potential (B) of extracellular vesicles. LMPEVs, freeze-dried monovarietal pomace extracellular vesicles; MPEVs, monovarietal pomace extracellular vesicles; PPEVs, plurivarietal pomace extracellular vesicles; WWEV, wastewater extracellular vesicles.

lipid mixture, which may contain an aqueous solution inside, tends to indicate that, at least, a fraction of the EV is not a remnant of membranes (plasma or thylakoid) but more likely naturally structured vesicles [51].

To characterize the size distribution of EV, samples were diluted with buffer and analyzed before and after filtration at 0.2 µm by DLS (Figure 7A). In the case of filtrated samples, a monomodal distribution (≃27 nm) was observed only for EV from PP. The other vesicles showed plurimodal distributions with main modes around 50–55 nm for WW and MP, whereas it was around 30 nm for EV from freeze-dried PPs. Larger particles (100–300 nm) were also present in MPEV and PPEV. These distributions correspond to the lower CryoTEM range scale. Non-filtrated samples showed higher diameters (Gaussian distribution, data not shown).

The zeta potential of filtrated samples was measured at five different pH values (7.2, 6, 5, 3, and 2) (Figure 7B). At neutral pH, zeta potential ranged between −40 and −10 mV. A decrease in the negative charge was observed and was positively correlated with the decrease of pH. Apparent isoelectric point was reached between pH = 2 and 3. A positive zeta potential was reached at pH 2 with a charge of around 15 mV for EV from PPs only, whereas most other vesicles are close to neutrality.

3.6 | DPPH-free radical scavenging assay for by-products and vesicular fractions

In order to evaluate their reducing capacity, a DPPH assay was performed for by-products and their EV vesicular fractions and compared with reference antioxidants (HT, Trolox) (Table 4). The radical scavenging activity of EV depended on their original by-product type and sample pre-treatment. EV isolated from WW (EC_{50} = 206 (2) mg L^{-1}) and freeze-dried MPs (EC_{50} = 239 (44) mg L^{-1}) showed the highest antioxidant activity, whereas the lowest was observed for EV obtained from MPs (EC_{50} = 474 (185) mg L^{-1}). Initial by-products showed lower scavenging activities than their corresponding EV. Knowing that these vesicles are strongly depleted in phenolics in comparison with their corresponding by-products, one can consider that their higher radical scavenging capacity is also due to the presence of other reducing compounds. For example, the membrane constituents of EV may have such an effect. Indeed, phospholipids are known to express radical scaveng-

TABLE 4 Free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl [DPPH]) of the different extracellular vesicles (EVs) comparing to by-products.

	EC_{50} (mg L^{-1})
WWEV	206 (2)
PPEV	290 (91)
MPEV	474 (185)
LMPEV	239 (44)
Wastewaters	394 (116)
Plurivarietal pomace	598 (60)
Monovarietal pomace	400 (26)
Freeze-dried monovarietal pomace	434 (16)
Hydroxytyrosol	5.1 (0)
Trolox	6.3 (3)

Abbreviations: LMPEVs, freeze-dried monovarietal pomace extracellular vesicles; MPEVs, monovarietal pomace extracellular vesicles; PPEVs, plurivarietal pomace extracellular vesicles; WWEV, wastewater extracellular vesicles.

ing activities or synergistic effect with tocopherols [52,53]. Similarly, galactolipids, in particular MGDG and DGDG, have a radical scavenging potential as shown by Terme et al. using the DPPH assay [54]. Proteins are also present in these fractions as shown by CLSM. These latter are known for their potential scavenging radical effect for which two possible mechanisms are described either the direct reaction between the amino acid at the end of the peptide chain with the free radicals or metal chelation due to the thiol group present in some amino acids [55,56].

4 | CONCLUSION

This study has shown that putative EVs are present in all olive oil by-products such as WWs or pomaces. Their size differed depending on the type of the by-products. Isolation of EV by centrifugation/ultracentrifugation may not be the ideal method in order to eliminate all the cell pollutants, as lipid characterization showed the presence of non-membranous lipid compounds. All were selectively enriched in polar lipids and selectively depleted in phenolic compounds. Polar lipids in EV were quite homogeneous, whatever the initial by-products and dominated by PC/PA, followed by DGDG/PE and then MGDG. Putative EVs isolated from WW have the highest content in phenolic compounds. WW was already the by-product having the highest amount of polar lipids. It is probably linked to the process used for its obtention. Such a process is indeed characterized by the addition of water during oil extraction, which probably favors the solubilization of polar lipids. Finally, when measuring their radical scavenging capacity by the DPPH assay, the higher efficiency of EV compared to their corresponding native by-products could be attributed to the presence of non-phenolic compounds such as polar lipids (phospholipids and galactolipids) or proteins. These putative olive EV should thus be investigated further as effective vehicles of antioxidants in which synergies and structural protection of activity can be tailored.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The author has provided the required data availability statement and, if applicable, included functional and accurate links to said data therein.

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