Journal of Food Composition and Analysis 134 (2024) 106494

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

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

Biophysical features of plant-derived nanovesicles: Focus on almonds

Carmen Santangelo [a,*], Enrico Binetti [b,d], S.N. Afifa Azman [c], Danilo Bondi [a], Virgilio Brunetti [d], Marco Farina [c], Cristina Purcaro [a], Lorenzo Marramiero [a], Rossella Di Raimo [e], Giulia Pietrangelo [e], Ester Sara Di Filippo [a], Tiziana Pietrangelo [a]

[a] Department of Neuroscience, Imaging and Clinical Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy
[b] Institute for Microelectronics and Microsystems, National Research Council of Italy (CNR-IMM), Lecce, Italy
[c] Department of Information Engineering, Marche Polytechnic University, Ancona, Italy
[d] Istituto Italiano di Tecnologia (IIT), Center for Bio-Molecular Nanotechnologies@UniLe, Via Barsanti, Arnesano, Lecce 73010, Italy
[e] ExoLab Italia, Tecnopolo d'Abruzzo, L'Aquila 67100, Italy

ARTICLE INFO

Keywords:
Nuts
Plant-derived extracellular vesicles
Exosome-like nanoparticles
Food components
Natural compounds
Vesicles isolation method
Density based ultracentrifugation
Microscopy visualization

ABSTRACT

Almond is a traditional food with established beneficial effects on health. Nothing is known about the presence of extracellular vesicles (EVs), recently isolated from other plant material, ingested with food, or as engineered bioactive nanovectors. Aiming to develop and optimize a method to isolate almonds derived nano vesicles (ADNVs), we tested different protocols on pure, blanched and roasted almonds, and investigated the resulting biophysical features.

The most feasible and valid method was the sucrose-based ultracentrifugation (DGUC). Size distribution ranged on average 100–200 nm. A negative zeta-potential in the range of –27 to –21 mV has been measured. Microscopy showed a spheroid morphology and preserved structural integrity of isolated ADNVs. Pure almond ADNVs showed the greater amount of TET8; PEN1 was also found, although cooking treatments resulted in a decrease.

Attention should be paid to sample managing during DGUC steps and to sample deposition and drying for microscopy procedures. Our findings contribute to enriching the hot issue on EVs research from edible sources, giving reason to their possible role in interspecies communication, and their exploitation for the delivery of bioactive compounds or therapeutics molecules. The potentiality of almonds' industrial residuals should be investigated in further research.

1. Introduction

The almond (*Prunus dulcis* (Mill.) D. A. Webb) is a historical and cultural food grated well known worldwide and produced natively in Central Asia reaching the Mediterranean regions over the ages (Casas-Agustench et al., 2011). Almonds are usually consumed raw, blanched or roasted, and processed to obtain derivatives such as milk, butter and oil (Grundy et al., 2016; Ouzir et al., 2021). Furthermore, they are used as ingredient in many dishes, representing a traditional food added to local recipes related to rural culture (Renna et al., 2021; Santangelo et al., 2022). The representative nutrients content of almonds, included in the U.S. Department of Agriculture (USDA) National Nutrient Database for Standard Reference, consists of lipids 71 %, protein 15 %, and carbohydrates 14 %; micronutrients are also present, and the mean greatest amount is for calcium, magnesium, potassium, and phosphorus (U.S. Department of Agriculture, 2023). The beneficial effects of almonds are mainly attributed to the lipid profile, represented above all by mono and polyunsaturated (60 % and 30 %) (Barreira et al., 2012), and to the fiber and micronutrient content. Almonds also represent a good source of phytochemicals such as proanthocyanidins, flavonoids, phenolic acids and phytosterols (Prgomet et al., 2017). The chemical composition of almonds confer to them beneficial properties on on humans such as 1) cardiovascular protection, glucose homeostasis, oxidative stress and neuroprotection, gut microbiota, and digestion and satiety (Barreca et al., 2020; Ouzir et al., 2021). The common heat treatment of roasting and blanching results in chemical and physical changes which affect the flavor, texture, color and aroma, and the consumer acceptance, on the basis of the geographical origin of the

* Correspondence to: University "G. d'Annunzio" Chieti – Pescara, Via dei Vestini, 31, Chieti 66100, Italy.
E-mail address: carmen.santangelo@unich.it (C. Santangelo).

https://doi.org/10.1016/j.jfca.2024.106494
Received 27 March 2024; Received in revised form 27 June 2024; Accepted 1 July 2024
Available online 8 July 2024
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cultivar (Oliveira et al., 2019). Furthermore, thermal and soaking treatments with salt can affect phytochemicals bioavailability, depending on temperature and times of process (Gonçalves et al., 2023; Kumari et al., 2020; Taylor et al., 2018).

The extracellular vesicles (EVs) derived from body fluids have been increasingly investigated in the last decade, (Hornung et al., 2020; Pietrangelo et al., 2023; Street et al., 2017) especially in mammalian systems (Bonsergent et al., 2021; Pol et al., 2012). The general terms "extracellular vesicles" is referred to the particles delimited by lipid-bilayer membrane, naturally released by all living cells and unable to replicate (Théry et al., 2018). The challenges in EVs research are related to the size diversity, biogenesis, target specificity, cell delivery, cargo loading and delivery of bioactive molecules, and therefore to their function in health and diseases (Margolis and Sadovsky, 2019). The International Society for Extracellular Vesicles (Pinedo et al., 2021) (ISEV; www.isev.org) identified different sizes of EVs: exosomes (30–120 nm), microvesicles (MVs) (100–1000 nm), and apoptotic bodies (1–6 μm in diameter) (see Vesiclepedia, www.microvesicles.org). EVs isolated from edible sources have a varied nomenclature, e.g., plant-derived exosome-like nanoparticles or nanovesicles (PDEN) and plant-derived vesicle-like nanoparticles (PDVLN). However, moving apart from the mammalian-derived EVs, the EVs isolated from plants are actually classified as "plant EVs" (PDV), replacing the term "exosomes", purified from the apoplastic fluid, and "plant-derived nanovesicles" (PDNV), referred to all vesicle isolated from the soft disruption of the plant tissues, containing a mixture of EVs and microvesicles (Pinedo et al., 2021). The FAO/INFOODS databases, named "FoodEVs", collect information about food EVs. Actually, information is limited to the four FAO groups of milk, starchy roots and tubers, nuts and seeds, and fruits (FAO, 2014. FAO/INFOODS databases, 2014. Food and Agriculture Organization of the United Nations (FAO). International Network of Food Data Systems (INFOODS): International food composition table/-database directory). PDNVs have been isolated in the last decade from the juice of various plants, of which ginger, grapefruits, grape, citrus, and broccoli. (Del Pozo-Acebo et al., 2022; Logozzi et al., 2021; Shinge et al., 2022). The isolation of PDNVs from roasted hot coffee beverages address towards the preliminary evidence of the vesicles resistance to heat (Kantarcıoğlu et al., 2023). The role of the PDNVs seem to be primary for plant-pathogen interactions, inter-kingdom communication and stress response (Subha et al., 2023). Recent studies emphasize the involvement of extracellular vesicles in interspecies communication, focusing on potential regulation of physiological processes of PDNVs ingested by humans through the diet every day. This leads primarily to the enhancement of the intestinal barrier, also impacting on the gut microbiota. Other highlighted functions were immunomodulation, antitumor effects, anti-inflammatory and anti-oxidative effect, regulation of metabolic dysregulation (improvement of lipids profile and of insulin sensitivity) (Kim and Kim, 2022; Kocholata et al., 2022), and the property to overcome the blood-brain barrier targeting neurological systems (Yang et al., 2018).

The biogenesis of the plants EVs is not well understood, but current researches suggest that the main classes of EVs are released in the plants apoplast following three different pathways: 1) the fusion of the Exocyst-Positive Organelles (EXPO) double membrane with the plasma membrane ("EXPO vesicles"), 2) the budding of the plasma membrane ("microvesicles or ectosome"), and 3) the fusion of the multivesicular bodies (MVBs) with the plasma membrane (intraluminal vesicles usually termed "exosomes") (Mahlein, 2016). Based on the EVs biogenesis, specific proteins were recently found as subclass markers. Specifically, the Exo70E2 protein was identified as a marker of the EXPO vesicles (Ding et al., 2014); on the other hand, the tetraspanin TET8, a plant analogue of the human exosomal marker CD63 (Théry et al., 2018), has been identified in MVBs vesicles (Chen and Cai, 2023; He et al., 2021). Lastly, the syntaxantin SYP121 (also named PEN1) was found in microvesicles (Rutter and Innes, 2017; Waghmare et al., 2018).

Several isolation techniques have been employed, involving the centrifugal force to obtain particles that differ in size and density (differential ultracentrifugation – UC), the use of density gradient centrifugation (DGC), and the combination of UC steps and density gradient centrifugation (DGUC) to separate high purity particles (Yakubovich et al., 2022). Other methods involved the combination of UC and Exo-Quick kit (Pieters et al., 2015). DGUC allows to yield better results in terms of EV fraction purity; UC and the number of EVs' proteins and RNA compared to classical UC (Shami-shah et al., 2023). UC combined with sucrose DGC has been largely used for isolating plant-derived nanovesicles (Sall and Flaviu, 2023). However, UC and DGUC share the disadvantages of requiring large volumes of samples and a long time. Interestingly, to reduce the time-consuming disadvantages while maintaining the advantages of DGUC in increased purity, cushioning effect for maintaining integrity, and separation of high-density protein contaminants, a one-step sucrose cushion ultracentrifugation has been validated (Gupta et al., 2018).

Visualizing EVs is a fundamental step of EVs research for a detailed morphological analysis. Electron microscopy is considered a standard imaging method for observing nanosized samples such as EVs, although samples need to be fixed and processed prior to imaging, resulting in possible cup shape from transmission electron microscopy (TEM) and saucer shape from scanning electron microscopy (SEM) (Chuo et al., 2018). The combination of electron microscopy and scanning probe microscopy (e.g. atomic force microscopy, AFM) allows to obtain detailed information of EVs' cross-sectional shape, height, and surface roughness.

Purified from edible sources, PDNVs constitute promising tools for drug-delivery systems, due to their biocompatibility, low toxicity and immunogenicity, and great availability from many sources (Nemati et al., 2022). Indeed, in addition to their intrinsic properties, these vesicles can be used as natural vectors intentionally altered to confer multiple functionalities and benefits as biotherapeutics are leading to a growing industry recognition and market share (He et al., 2023). However, PDNVs research is still in its infancy, and increased and rigorous evidences are needed to foster its role in biomedicine (Pinedo et al., 2021). To the best of our knowledge, no evidence exists on PDNVs from almonds.

1.1. Aims of the study

In this study, we aimed to develop and optimize a method to isolate PDNVs from almonds (ADNVs) and analyze them by means of biophysical features. We tested different protocols based on UC steps, syringe filters, and DGUC steps. We also aimed to evaluate, if any, the effects of cooking and preparation of almonds on isolated EVs.

2. Materials and methods

2.1. Comprehensive procedure of ADNVs characterization

The first step was to determine a feasible and valid method for isolating ADNVs, thus obtaining characterizable samples. Several sources and isolation methods were empirically tested in our Labs (see supplementary materials).

First, we tested the standard UC protocol on almond milk, which is easier to handle. To increase the purity of the samples, we used sucrose-based DGUC. We observed values from average size, polydispersity index, and multi-angle peaks. Using 0.22 or 0.45 μm filters the vesicles' diameters were greater and overdispersed. We then tested sucrose-based DGUC with the one-step method (Gupta et al., 2018) and with multi-level approaches, maintaining a similar time course thus avoiding the overnight procedure. For what concerns this latter procedure, we observed that the 90-minutes long multi-level DGUC was not enough for effectively separating different EVs.

2.2. ADNVs isolation

After the preliminary phase, ADNVs were isolated by DGUC steps as follows:

1) Prior mixing in PBS (1x, pH of 7.39) (PBS) and grounding at the highest speed for 3 min, on ice, using Ultra-Turrax 131 T25 Basic (Basic IKA- Werke, Staufen, Germany)
2) Centrifuge at relative centrifugal force (RCF) of 400 x g, 15 min 4°C
3) Surface fat removal and resuspension w/ PBS
4) Centrifuge at RCF 800 x g, 15 min 4°C on remaining sample to recover the surnatant
5) Centrifuge the surnatant at RCF 10,000 x g, 45 min 4°C, surface fat removal
6) Add 1 ml of sucrose cushion 30 % on fresh tube and add carefully the collected surnatant
7) Centrifuge at RCF 100,000 x g, 90 min 4°C
8) Surnatant remotion maintaining cushion volume
9) Add PBS to reach the final volume and mix the sample by turning it upside down
10) Centrifuge at RCF 100,000 x g, 60 min 4°C
11) Resuspension w/ 120 μL of PBS

ADNVs were isolated from whole homogenates of commercially available edible dried almonds (*Prunus amygdalus* var. *dulcis*). Multiple centrifugation steps were performed on 1) normal dried almonds, 2) blanched almonds (85–100°C hot water x 2–5 min), 3) roasted almonds (130 °C x 5 min), 4) normal and roasted almonds soaked for 12 h, according to reported method (Kumari et al., 2020) and 5) "Bocconotti" (Italian traditional almond dessert) (Santangelo et al., 2022). Following isolation, EVs were characterized by size distribution, zeta potential, morphology, topography, and presence of the PDNVs surface markers.

2.3. Quality control of isolated vesicles

After isolation, the nucleic acid-to-protein ratio (NPr) of ADNVs was estimated by UV absorbance at 260 nm and 280 nm, respectively, by UV–vis spectrophotometry in a Nanophotometer NP80 (Implen, Germany), calibrating the instrument using sample elution buffer Blank Control™ (Implen, Germany) before the test. The NPr served as a means of evaluating the purity of EVs samples, since this approach is sensitive and non-destructive. It reflects the relative composition of nucleic acid and protein because of low-UV-absorbing phospholipids that mainly constitute vesicles' membranes, thereby serving to evaluate the purity and relative protein or nucleic acid contamination, and requiring only small amounts of material (Sun et al., 2021). The samples were stored at −80 °C until the characterization.

2.4. Atomic Force Microscopy for ADNVs size distribution and surface roughness

Morphology was evaluated by scanning atomic force microscopy (AFM) (NT-MDT Solver Pro P-47). The measurements were collected in semi-contact mode by using a probe with a resonant frequency of 140 kHz and a spring constant of 3.5 N/m (HA_NC ETALON, NT-MDT). The vesicles from the scanning data were marked as grains by thresholding algorithms, to distinguish them from the background surface. The marked region is regarded as the area of interest for statistical evaluations, and the quantities are expressed using integrals of the height distribution function with some power of height. This post-processing was done by Nova Px, an interface of the program that controls scanning probe microscopes that also allows for analysis of scanning probe microscopy measurements. The surface roughness was reported as 1) the variance of height distribution, that is the mean square (RMS) of height irregularities (Sq), computed from the 2nd central moment of data values, 2) the mean value for each grain separately (RMS grain-wise), and 3) the mean roughness (Sa) value of height irregularities.

2.5. Dynamic light scattering and zeta potential for ADNVs size and charge distribution

The average hydrodynamic diameter measurements were performed on the Zetasizer Nano instrument (Malvern, United Kingdom) equipped with a 10 mW He–Ne laser operating at 633 nm, fixed scattering angle of 173°. PBS-EVs solutions were diluted 20x before being loaded in 1-cm polystyrene cuvette for Dynamic Light Scattering (DLS) measurements, while they were diluted 40x before being loaded in Malvern DTS1070 cells for Zeta Potential (ZP) measurements. Three measurements have been carried out for each sample.

2.6. Nanoparticle tracking analysis (NTA) for ADNVs size distribution and particle concentration

Nanoparticle Tracking Analysis (NTA) from Malvern (NanoSight NS300, Worcestershire, UK) was used. Three videos of 60 s duration were taken. Data were analyzed using the NTA 3.0 software (Malvern Instruments), optimized to first identify and then track each particle on a frame-by-frame basis. The Stokes–Einstein equation: $D^\circ = kT/6\pi\eta r$, where D° is the diffusion coefficient, was used to track the Brownian motion of each particle; $kT/6\pi\eta r = f0$ is the frictional coefficient of the particle, for the special case of a spherical particle of radius r moving at a uniform velocity in a continuous fluid of viscosity h; k is Boltzmann's constant, and T is the absolute temperature.

2.7. Transmission electron microscopy for ADNVs morphology and structure visualization

Transmission Electron Microscopy (TEM) analysis was performed with a JEOL JEM-1011 transmission electron microscope at 100 kV operating voltage, equipped with a 7.1 megapixel CCD camera (Orius SC1000, Gatan, Pleasanton, CA). Purified EVs solution were simply drop-casted on 200 mesh formvar supported copper grids, dried in air and immediately analyzed.

2.8. Western Blotting (WB) for ADNVs type identification

The samples were loaded on BoltTM 4–12 % Bis-Tris Plus Gels (Invitrogen REF NW04120BOX), run at 150 V for 35 min and transferred to a nitrocellulose membrane with iBlot 2 Dry Blotting System (Thermo Fisher Scientific, Cat # IB21001). The membrane was blocked for 1 h at room temperature (RT) with 5 % Skim-milk (Sigma–Aldrich) in TBS buffer supplemented with 0.1 % Tween-20 (Sigma–Aldrich). The membrane was incubated overnight at 4°C with primary antibodies TET8 (PHY1491S, PhytoAB Inc.) at 1:1000, and SYP121 (PHY2912S, PhytoAB Inc.) at 1:1000. After incubation with Goat Anti Rabbit IgG H&L (PHY6000, PhytoAB Inc.) at 1:5000 for 1 h at RT, the immunodetection was performed with the SuperSignal West Pico PLUS chemiluminescent substrate (thermoscientific REF 34577). Images were obtained and edited on the UVITEC machine (Cambridge).

3. Results and discussion

ADNVs were isolated as previously described. A representative appearance of the sample is shown in Fig. 1.

3.1. Quality control

The quality control of the preliminary procedures was carried out using the Nanophotometer NP80. The 260/280 ratio was in the range of 0.31–0.83 from 1 μl sampling solution.

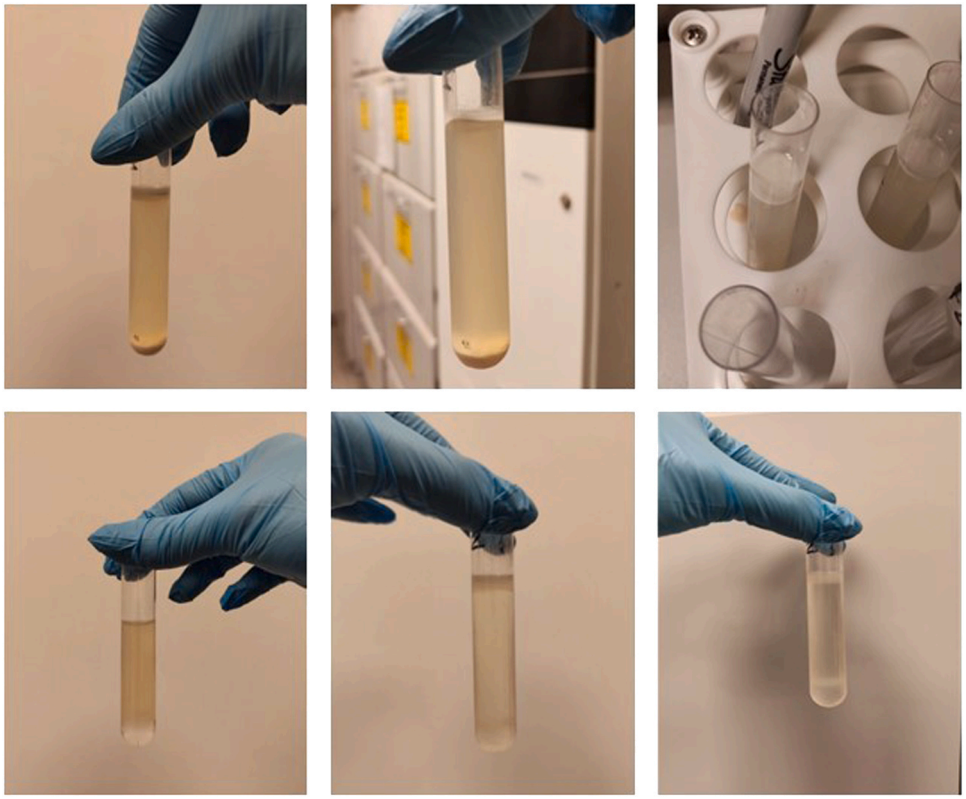

Fig. 1. Representative images of DGUC steps for isolating ADNVs.

3.2. Dynamic light scattering and zeta potential analysis

DLS analysis (Fig. 2) revealed the presence of a stable suspension of particles with a size distribution around 170 nm. Such result is consistent with how previously reported for some PDEVs (Kilasoniya et al., 2023), peculiar compared to the size distribution of EVs from ginger and citrus (Di Gioia et al., 2020).

A negative zeta-potential (ZP) in the range of –27 to –21 mV has been measured for all the ADNVs dispersions. Such values are similar to zeta potentials measured in urinary EVs (Pietrangelo et al., 2023) and in a similar range of other evidence on PDNVs' surface charge (Di Gioia et al., 2020). ZP is determined by the surface charge of vesicles and indicates their colloidal stability which influences particle–particle and particle–medium interactions, i.e., the closer the ZP to zero, the higher the tendency to aggregate.

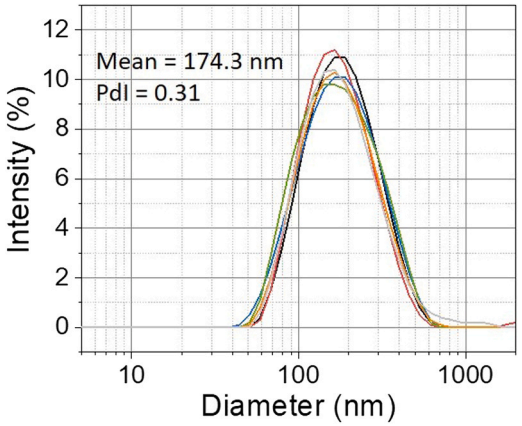

Fig. 2. DLS analysis of ADNVs. Average size-distributions determined by Zetasizer Nano instrument. Lines represent replicates.

3.3. Nanoparticle tracking analysis (NTA)

To obtain more stable results, we determined the mean size and the particle concentration throught NTA for normal dried almond, almonds roasted and soaked, roasted almonds, and blanched almonds (Fig. 3). Mean size was found compatible to PDNVs (Mu et al., 2023). The yeld of ADNVs from 10 g of starting samples was comparable to how previously obtained from 100 ml of tomato juice (Kilasoniya et al., 2023), with vesicles concentration ranging from 5.5 and 7.7 $\times 10^{11}$. Particles concentration from blanched almonds was higher than other samples (1.2 $\times 10^{12}$), similar to vesicles isolated from grapefruit juice (Kilasoniya et al., 2023).

3.4. Atomic force and transmission electron microscopy

Fig. 4 shows the representative surface topology estimated by AFM, both unmasked and masked, including several metrics of surface roughness. Fig. 4 shows the morphological characterization of ADNVs.

Images from TEM show a round shape and a preserved morphology of ADNVs isolated from normal dried almonds through DGUC (Fig. 5).

Previously morphological characterization of grapefruits and tomatoes juice samples showed spherical or oval-shaped nanovesicles (Kilasoniya et al., 2023). From microscopy, we demonstrated the spheroid morphology of isolated ADNVs, with preserved structural integrity and a diameter plausible with the average as reported by DLS. All the three metrics of roughness had values included in those ranges we previously evidenced in urinary EVs, more similar to that we found in small EVs from triathletes than larger EVs from control participants (Pietrangelo et al., 2023).

3.5. Western blot

The first series of western blotting (WB)aimed to detect the presence of TET8 on ADNVs isolated from different matrices and through

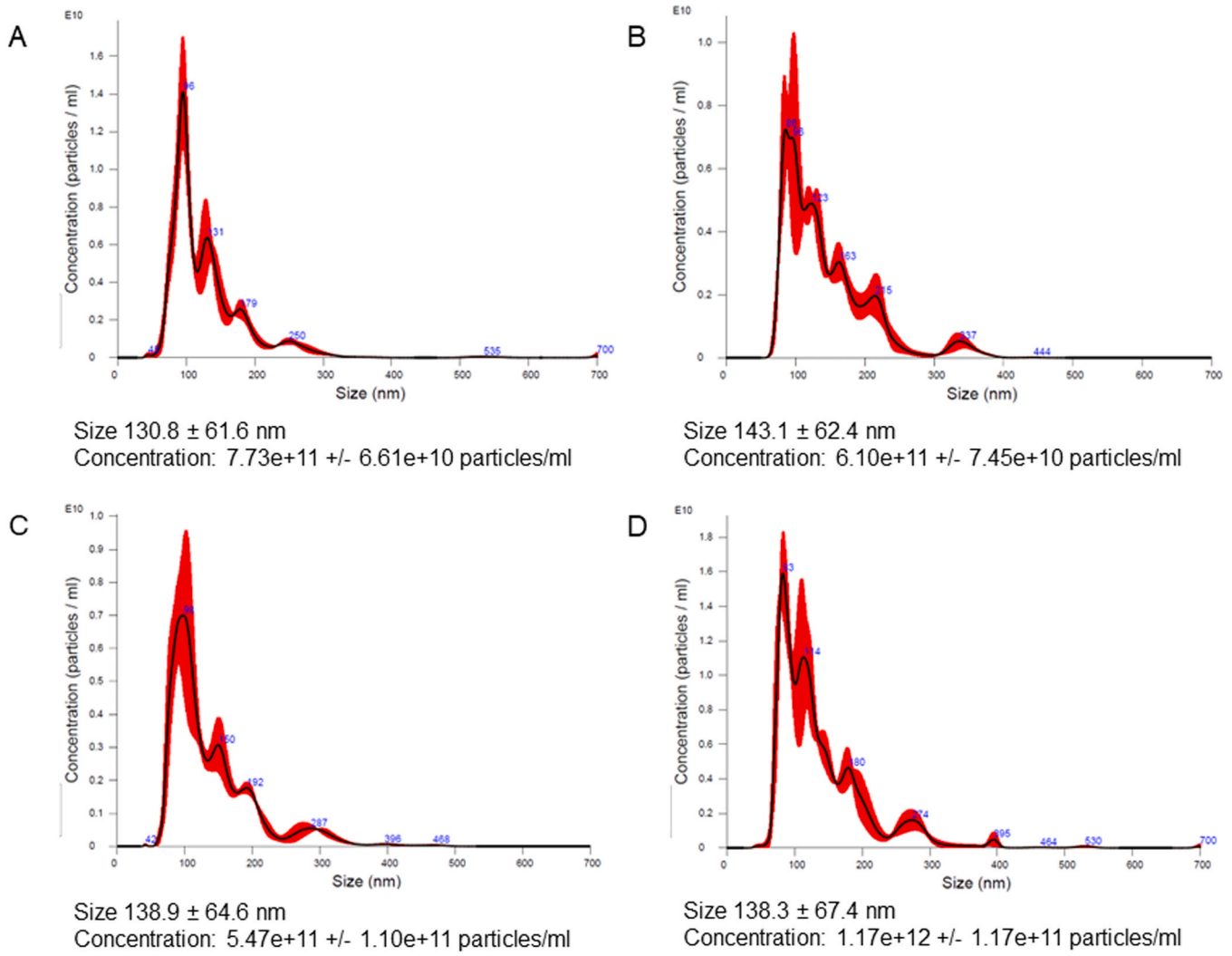

Fig. 3. NTA of ADNVs isolated through DGUC. Average size-distributions and particle concentration. Data are reported as mean value ± standard deviation. A: normal dried almonds; B: roasted and soaked almonds; C: roasted almonds; D: blenched almonds.

different protocols. As shown in Fig. 6, the tetraspanin TET8 was absent in the recipe "Bocconotti", whilst it was slightly present in almond milk and pure almonds, particularly in DGUC protocols (first line). Although the band was not precise, it is clearly identifiable from the figure that in the sample of pure almonds isolated with the DGUC protocol the presence of TET8 was largely greater than in the other samples analyzed. Moreover, performing WB analysis on DGUC isolated sample, we detected TET8 greatly represented after soaking of normal dried almond and blenching; TET8 was slight present in roasted sample and well detected in soaked roasted almond (second line). The second step used was aimed to detect syntaxantin SYP121 (PEN1) in ADNVs isolated through DGUC protocols from almonds; different cooking treatments were implemented, resulting in slight WB bands observable in pure almonds, as well in almonds after combined soaking and roasting.

Collectively, evidence suggests that PEN1-positive and TET8-positive vesicles are two subtypes of PDNVs: since TET8 is structurally alike animal CD63, TET8-positive nanovesicles partially colocalize with the multivesicular bodies' marker ARA6 and have been observed outside of cells, TET8-positive nanovesicles are considered as plant exosomes (Liu et al., 2021; Nemati et al., 2022). Our findings revealed that almonds express preferentially TET-8 positive, rather than PEN1-positive, nanovesicles isolated with sucrose-DGUC. Considering DGUC overcomes UC in isolating exosomes (Greening et al., 2015; Gupta et al., 2018), we conclude that one-step sucrose-cushion UC likely resulted in preferential isolation of exosomal subtype of ADNVs.

4. Critical steps, alternatives, troubleshooting, caution/ warnings

Problems may be encountered in the removal of the lipid layer during the isolation, which occurs at all centrifugation steps, which can be conducted with the use of a little laboratory settle scoop or spoon; an accurate remotion prevents further problems due to those lipid layers affecting pipette tips. The use of filters such as 0.22 μm for size-exclusion procedures may produce agglomerates, which can affect any further analysis for size distribution and topography. In AFM it is necessary to deposit samples, and to this aim the original PBS solution is diluted in 100 ml distilled water, and then deposited on a mica layer and left dry; the dilution requires a careful tradeoff, since a large number of vesicles per unit volume may result in multi-layer deposition or even formation of agglomerates, while a small number may result in hard to find vesicles in the scanning area, necessarily limited to a few microns. Evaporation of distilled water is necessary to avoid tip sticking or a water meniscus during AFM scan; however, during this time, some environmental contaminants may deposit over samples. Crystallization of PBS residual is also a possible phenomenon during microscopy procedures, with the appearance of shapes attributable to this event. We have evaluated the possibility of performing AFM in liquid, but in this case specific

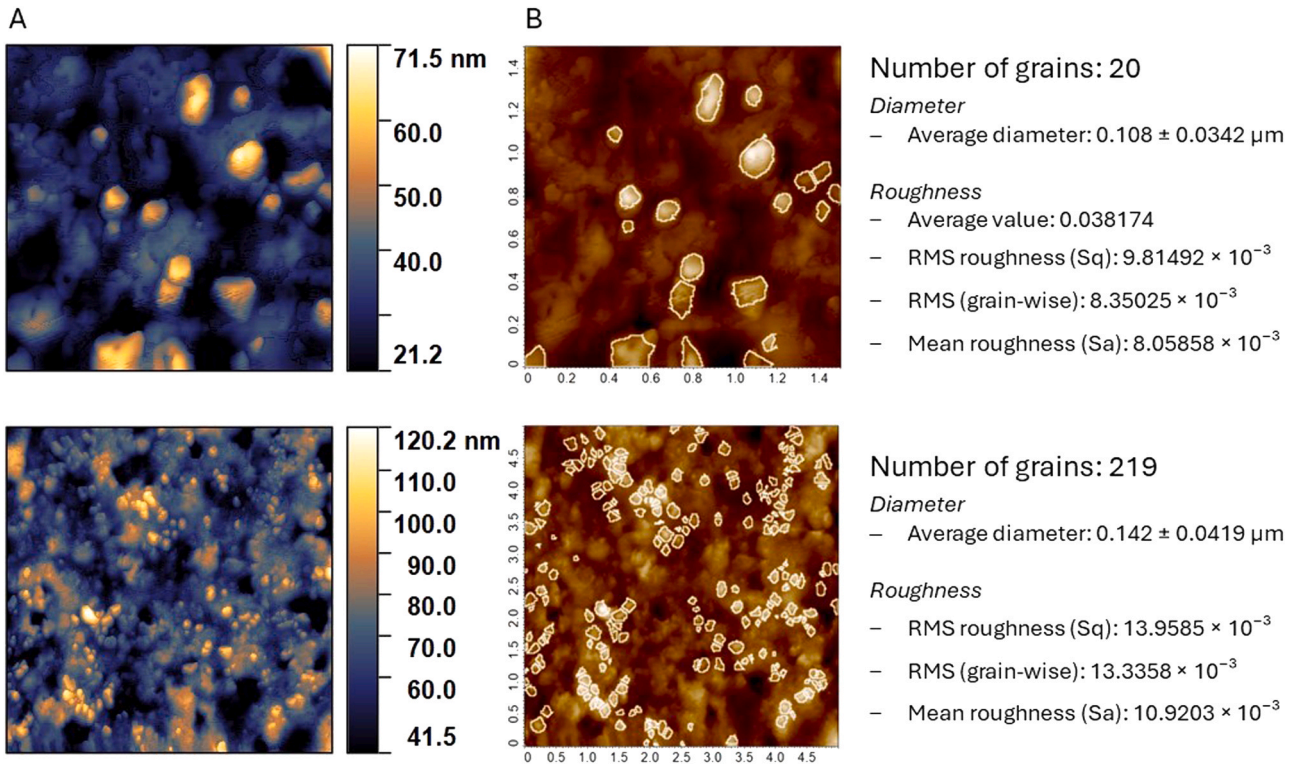

Fig. 4. Representative images of topography derived from AFM analysis of ADNVs from roasted almonds. Diameters are reported as mean value ± standard deviation. Panel A: non-masked images; the colorimetric legend refers to the height of the object. Panel B: scanned vesicles marked as grains. RMS Sq: mean square of height irregularities (Sq); RMS (grain-wise): mean value for each grain separately; mean roughness (Sa): mean value of height irregularities.

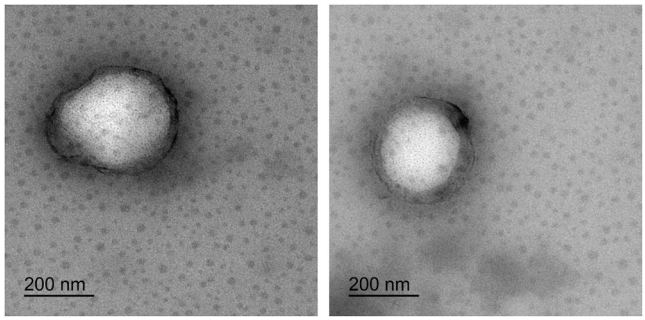

Fig. 5. Representative ADNVs micrographs acquired by TEM. Vesicles were isolated from normal raw almond through DGUC.

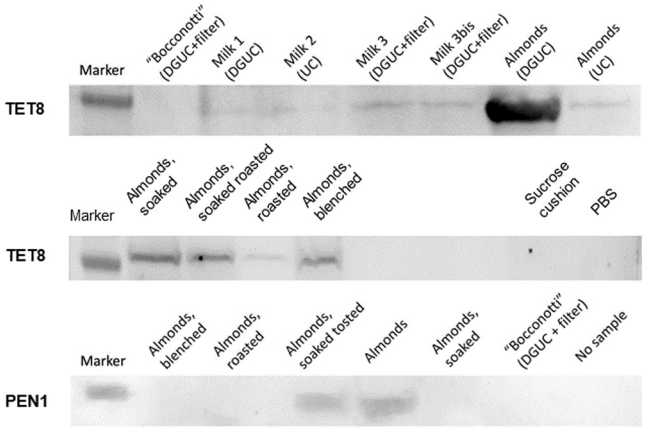

Fig. 6. Western blot for vesicles type identification.

immunostaining procedures should be developed to keep vesicles on mica and prevent them from floating, beyond the scope of this paper. During NTA, we detected particles in both the MilliQ and PBS solution used to dilute and to clean between each sample analysis. Attention should be paid to avoid compromission of the analysis accuracy. We suggest to use fresh materials and to filter them. When using NTA, PBS washing should be conducted interspersed between each sample as to avoid cross-contamination. As well as for AFM analysis, the sample preparation for TEM requires solution deposition and drying with the occurrence of mentioned problems. In addition, dried NVs can collapse in the TEM vacuum chamber, making identification impossible. Attention should be paid in comparing ZP between different PDNV sources from different protocols of measurement; indeed, the physico-chemical characteristics of the medium influence these measures, as ZP became more negative as pH increase; instead, higher concentrations of multivalent ions, such as Ca^{2+}, Al^{3+}, and phosphate, decrease the negative value of ZP; moreover, the presence of detergent decreases the negative value of ZP, as reported in (Midekessa et al., 2020).

5. Conclusions and perspectives

Our findings provide evidence for the existence of nanovesicles from almonds, adding this edible source along with other sources such as milk, starchy roots and tubers, vegetables, seeds, fruits, and other nuts. In addition to the biological evidence, almonds may constitute an exploitable source for the delivery of bioactive compounds and drugs, due to the possibility to fuse biotechnology with organic agriculture to overcome some current challenges in drug delivery systems after cargo-loading methods (Orefice et al., 2023). We extend the argument in favor of the PDNVs' resistance to heat, as previously demonstrated for roasted hot coffee beverages (Kantarcıoğlu et al., 2023), herein demonstrating the presence of almonds' PDNVs after hot treatments. This and other arguments support the use of EVs from edible sources and food as ideal nanovectors for bioactive compounds or therapeutic molecules, given

they do not derive from intensive agriculture or other procedures that include toxicants or unwanted materials, since EVs represent a way of biological entities for eliminating extracellularly toxic molecules (Logozzi et al., 2022).

Specific analyses may be conducted to depict the spontaneous formation of a putative protein corona as a proteinaceous layer on PDNV surface, as already demonstrated for human exosomes (Heidarzadeh et al., 2023) and synthetic nanoparticles (Mahmoudi et al., 2023), its components and its effect on biophysical and biochemical properties.

Vesicles isolation and characterization from almond peel and kernel could be carried out to assess the potential of industrial almond processing residuals.

Funding

One author (C.S.) has received funding from the Fund for Development and Cohesion (FSC), "Research and Innovation 2015–2017", supporting the doctoral program of Medical Biotechnologies at University "G. d'Annunzio" of Chieti (Italy). This research was supported by the Italian Ministry of Health (Ministero della Salute) MOOD Project- Grant D73C22001670006.

CRediT authorship contribution statement

Marco Farina: Writing – review & editing, Resources. **Cristina Purcaro:** Writing – review & editing, Methodology. **Danilo Bondi:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Virgilio Brunetti:** Writing – review & editing, Resources. **Enrico Binetti:** Writing – original draft, Visualization, Investigation, Formal analysis. **S N Afifa Azman:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ester Sara Di Filippo:** Writing – review & editing. **Tiziana Pietrangelo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Carmen Santangelo:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Giulia Pietrangelo:** Writing – review & editing, Visualization. **Lorenzo Marramiero:** Writing – review & editing, Methodology. **Rossella Di Raimo:** Writing – review & editing, Visualization.

Declaration of Competing Interest

No conflict of interest exists.

Data Availability

Data will be made available on request.

Acknowledgments

Authors thank Prof. Christian Celia and his group's members for the help and constructive discussion in identifying the pitfalls of EVs isolation procedures.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106494.

References

Barreca, D., Nabavi, S.M., Sureda, A., Rasekhian, M., Raciti, R., Silva, A.S., Annunziata, G., Arnone, A., Tenore, G.C., Süntar, İ., Mandalari, G., 2020. Almonds (Prunus dulcis Mill. D. A. Webb): a source of nutrients and health-promoting compounds. Nutrients *12* (3), 672 https://doi.org/10.3390/nu12030672.

Barreira, J.C.M., Casal, S., Ferreira, I.C.F.R., Peres, A.M., Pereira, J.A., Oliveira, M.B.P.P., 2012. Supervised chemical pattern recognition in almond (Prunus dulcis) Portuguese PDO cultivars: PCA- and LDA-based triennial study. J. Agric. Food Chem. *60* (38), 9697–9704. https://doi.org/10.1021/jf301402t.

Bonsergent, E., Grisard, E., Buchrieser, J., Schwartz, O., Théry, C., Lavieu, G., 2021. Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells. Nat. Commun. *12* (1), 1864. https://doi.org/10.1038/s41467-021-22126-y.

Casas-Agustench, P., Salas-Huetos, A., Salas-Salvadó, J., 2011. Mediterranean nuts: origins, ancient medicinal benefits and symbolism. Public Health Nutr. *14* (12A), 2296–2301. https://doi.org/10.1017/S1368980011002540.

Chen, Y.-X., Cai, Q., 2023. Plant exosome-like nanovesicles and their role in the innovative delivery of RNA therapeutics. Biomedicines *11* (7), 1806. https://doi.org/10.3390/biomedicines11071806.

Chuo, S.T.-Y., Chien, J.C.-Y., Lai, C.P.-K., 2018. Imaging extracellular vesicles: current and emerging methods. J. Biomed. Sci. *25* (1), 91. https://doi.org/10.1186/s12929-018-0494-5.

Del Pozo-Acebo, L., López de Las Hazas, M.-C., Tomé-Carneiro, J., Del Saz-Lara, A., Gil-Zamorano, J., Balaguer, L., Chapado, L.A., Busto, R., Visioli, F., Dávalos, A., 2022. Therapeutic potential of broccoli-derived extracellular vesicles as nanocarriers of exogenous miRNAs. Pharmacol. Res. *185*, 106472 https://doi.org/10.1016/j.phrs.2022.106472.

Di Gioia, S., Hossain, M.N., Conese, M., 2020. Biological properties and therapeutic effects of plant-derived nanovesicles. Open Med. *15* (1), 1096–1122. https://doi.org/10.1515/med-2020-0160.

Ding, Y., Wang, J., Chun Lai, J.H., Ling Chan, V.H., Wang, X., Cai, Y., Tan, X., Bao, Y., Xia, J., Robinson, D.G., Jiang, L., 2014. Exo70E2 is essential for exocyst subunit recruitment and EXPO formation in both plants and animals. Mol. Biol. Cell *25* (3), 412–426. https://doi.org/10.1091/mbc.e13-10-0586.

Gonçalves, B., Pinto, T., Aires, A., Morais, M.C., Bacelar, E., Anjos, R., Ferreira-Cardoso, J., Oliveira, I., Vilela, A., Cosme, F., 2023. Composition of nuts and their potential health benefits—an overview. Foods *12* (5), 942. https://doi.org/10.3390/foods12050942.

Greening, D.W., Xu, R., Ji, H., Tauro, B.J., Simpson, R.J., 2015. A protocol for exosome isolation and characterization: Evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods Mol. Biol. (Clifton, N. J.) *1295*, 179–209. https://doi.org/10.1007/978-1-4939-2550-6_15.

Grundy, M.M., Lapsley, K., Ellis, P.R., 2016. A review of the impact of processing on nutrient bioaccessibility and digestion of almonds. Int. J. Food Sci. Technol. *51* (9), 1937–1946. https://doi.org/10.1111/ijfs.13192.

Gupta, S., Rawat, S., Arora, V., Kottarath, S.K., Dinda, A.K., Vaishnav, P.K., Nayak, B., Mohanty, S., 2018. An improvised one-step sucrose cushion ultracentrifugation method for exosome isolation from culture supernatants of mesenchymal stem cells. Stem Cell Res. Ther. *9* (1), 180. https://doi.org/10.1186/s13287-018-0923-0.

He, B., Cai, Q., Qiao, L., Huang, C.-Y., Wang, S., Miao, W., Ha, T., Wang, Y., Jin, H., 2021. RNA-binding proteins contribute to small RNA loading in plant extracellular vesicles. Nat. Plants *7* (3), 342–352. https://doi.org/10.1038/s41477-021-00863-8.

He, W., Zheng, S., Zhang, H., Gao, B., Jin, J., Zhang, M., He, Q., 2023. Plant-derived vesicle-like nanoparticles: clinical application exploration and challenges. Int. J. Nanomed. *18*, 5671–5683. https://doi.org/10.2147/IJN.S428647.

Heidarzadeh, M., Zarebkohan, A., Rahbarghazi, R., Sokullu, E., 2023. Protein corona and exosomes: new challenges and prospects. Cell Commun. Signal. *21* (1), 64. https://doi.org/10.1186/s12964-023-01089-1.

Hornung, S., Dutta, S., Bitan, G., 2020. CNS-derived blood exosomes as a promising source of biomarkers: opportunities and challenges. Front. Mol. Neurosci. *13* https://doi.org/10.3389/fnmol.2020.00038.

Kantarcıoğlu, M., Yıldırım, G., Oktar, P.A., Yanbakan, S., Büşra Özer, Z., Sarıca, D.Y., Taşdelen, S., Bayrak, E., Akın Balı, D.F., Öztürk, S., Can Akçalı, K., Ezer, Ü., Kürekçi, A.E., 2023. Coffee-derived exosome-like nanoparticles: are they the secret heroes? Turk. J. Gastroenterol. *34* (2), 161–169. https://doi.org/10.5152/tjg.2022.21895.

Kilasoniya, A., Garaeva, L., Shtam, T., Spitsyna, A., Putevich, E., Moreno-Chamba, B., Salazar-Bermeo, J., Komarova, E., Malek, A., Valero, M., Saura, D., 2023. Potential of plant exosome vesicles from grapefruit (Citrus × paradisi) and tomato (Solanum lycopersicum) juices as functional ingredients and targeted drug delivery vehicles. Antioxid. (Basel, Switz.) *12* (4), 943. https://doi.org/10.3390/antiox12040943.

Kim, S.Q., Kim, K.-H., 2022. Emergence of edible plant-derived nanovesicles as functional food components and nanocarriers for therapeutics delivery: potentials in human health and disease. Cells *11* (14), 2232. https://doi.org/10.3390/cells11142232.

Kocholata, M., Maly, J., Martinec, J., Auer Malinska, H., 2022. Plant extracellular vesicles and their potential in human health research, the practical approach. Physiol. Res. *71* (3), 327–339. https://doi.org/10.33549/physiolres.934886.

Kumari, S., Gray, A.R., Webster, K., Bailey, K., Reid, M., Kelvin, K.A.H., Tey, S.L., Chisholm, A., Brown, R.C., 2020. Does «activating» nuts affect nutrient bioavailability? Food Chem. *319*, 126529 https://doi.org/10.1016/j.foodchem.2020.126529.

Liu, G., Kang, G., Wang, S., Huang, Y., Cai, Q., 2021. Extracellular vesicles: emerging players in plant defense against pathogens. Front. Plant Sci. *12* https://doi.org/10.3389/fpls.2021.757925.

Logozzi, M., Di Raimo, R., Mizzoni, D., Fais, S., 2021. Nanovesicles from organic agriculture-derived fruits and vegetables: characterization and functional antioxidant content. Int. J. Mol. Sci. *22* (15), 8170. https://doi.org/10.3390/ijms22158170.

Logozzi, M., Di Raimo, R., Mizzoni, D., Fais, S., 2022. The potentiality of plant-derived nanovesicles in human health—a comparison with human exosomes and artificial nanoparticles. Articolo 9 Int. J. Mol. Sci. *23* (9). https://doi.org/10.3390/ijms23094919.

Mahlein, A.-K., 2016. Plant disease detection by imaging sensors – parallels and specific demands for precision agriculture and plant phenotyping. Plant Dis. *100* (2), 241–251. https://doi.org/10.1094/PDIS-03-15-0340-FE.

Mahmoudi, M., Landry, M.P., Moore, A., Coreas, R., 2023. The protein corona from nanomedicine to environmental science. Nat. Rev. Mater. *8* (7), 422–438. https://doi.org/10.1038/s41578-023-00552-2.

Margolis, L., Sadovsky, Y., 2019. The biology of extracellular vesicles: the known unknowns. PLOS Biol. *17*, e3000363 https://doi.org/10.1371/journal.pbio.3000363.

Midekessa, G., Godakumara, K., Ord, J., Viil, J., Lättekivi, F., Dissanayake, K., Kopanchuk, S., Rinken, A., Andronowska, A., Bhattacharjee, S., Rinken, T., Fazeli, A., 2020. Zeta potential of extracellular vesicles: toward understanding the attributes that determine colloidal stability. ACS Omega *5* (27), 16701–16710. https://doi.org/10.1021/acsomega.0c01582.

Mu, N., Li, J., Zeng, L., You, J., Li, R., Qin, A., Liu, X., Yan, F., Zhou, Z., 2023. Plant-derived exosome-like nanovesicles: current progress and prospects. Int. J. Nanomed. *18*, 4987–5009. https://doi.org/10.2147/IJN.S420748.

Nemati, M., Singh, B., Mir, R.A., Nemati, M., Babaei, A., Ahmadi, M., Rasmi, Y., Golezani, A.G., Rezaie, J., 2022. Plant-derived extracellular vesicles: a novel nanomedicine approach with advantages and challenges. Cell Commun. Signal.: CCS *20* (1), 69. https://doi.org/10.1186/s12964-022-00889-1.

Oliveira, I., Meyer, A.S., Afonso, S., Aires, A., Goufo, P., Trindade, H., Gonçalves, B., 2019. Phenolic and fatty acid profiles, α-tocopherol and sucrose contents, and antioxidant capacities of understudied Portuguese almond cultivars. J. Food Biochem. *43* (7), e12887 https://doi.org/10.1111/jfbc.12887.

Orefice, N.S., Di Raimo, R., Mizzoni, D., Logozzi, M., Fais, S., 2023. Purposing plant-derived exosomes-like nanovesicles for drug delivery: patents and literature review. Expert Opin. Ther. Pat. *33* (2), 89–100. https://doi.org/10.1080/13543776.2023.2195093.

Ouzir, M., Bernoussi, S.E., Tabyaoui, M., Taghzouti, K., 2021. Almond oil: a comprehensive review of chemical composition, extraction methods, preservation conditions, potential health benefits, and safety. Compr. Rev. Food Sci. Food Saf. *20* (4), 3344–3387. https://doi.org/10.1111/1541-4337.12752.

Pieters, B.C.H., Arntz, O.J., Bennink, M.B., Broeren, M.G.A., van Caam, A.P.M., Koenders, M.I., van Lent, P.L.E.M., van den Berg, W.B., de Vries, M., van der Kraan, P.M., van de Loo, F.A.J., 2015. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-β. PloS One *10* (3), e0121123. https://doi.org/10.1371/journal.pone.0121123.

Pietrangelo, T., Santangelo, C., Bondi, D., Cocci, P., Piccinelli, R., Piacenza, F., Rosato, E., Azman, S.N.A., Binetti, E., Farina, M., Locatelli, M., Brunetti, V., Le Donne, C., Marramiero, L., Di Filippo, E.S., Verratti, V., Fulle, S., Scollo, V., Palermo, F., 2023. Endurance-dependent urinary extracellular vesicle signature: shape, metabolic miRNAs, and purine content distinguish triathletes from inactive people. Pflügers Arch. - Eur. J. Physiol. *475* (6), 691–709. https://doi.org/10.1007/s00424-023-02815-x.

Pinedo, M., de la Canal, L., de Marcos Lousa, C., 2021. A call for Rigor and standardization in plant extracellular vesicle research. J. Extracell. Vesicles *10* (6), e12048. https://doi.org/10.1002/jev2.12048.

Pol, E., van der, Böing, A.N., Harrison, P., Sturk, A., Nieuwland, R., 2012. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol. Rev. *64* (3), 676–705. https://doi.org/10.1124/pr.112.005983.

Prgomet, I., Gonçalves, B., Domínguez-Perles, R., Pascual-Seva, N., Barros, A.I.R.N.A., 2017. Valorization challenges to almond residues: phytochemical composition and functional application. Mol. (Basel, Switz.) *22* (10), 1774. https://doi.org/10.3390/molecules22101774.

Renna, M., Montesano, F.F., Serio, F., Gonnella, M., 2021. Chapter 5—the mediterranean diet between traditional foods and human health through culinary examples. In: Galanakis (A c. Di), C.M. (Ed.), Gastronomy and Food Science. Academic Press, pp. 75–99. https://doi.org/10.1016/B978-0-12-820057-5.00005-4.

Rutter, B.D., Innes, R.W., 2017. Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. Plant Physiol. *173* (1), 728–741. https://doi.org/10.1104/pp.16.01253.

Sall, I.M., Flaviu, T.A., 2023. Plant and mammalian-derived extracellular vesicles: a new therapeutic approach for the future. Front. Bioeng. Biotechnol. *11* https://doi.org/10.3389/fbioe.2023.1215650.

Santangelo, C., Mandracchia, F., Bondi, D., Piccinelli, R., Catasta, G., Llauradó, E., Tarro, L., Verratti, V., Cichelli, A., Sette, S., Pietrangelo, T., 2022. Traditional dishes, online tools, and public engagement: a feasible and scalable method to evaluate local recipes on nutritional content, sustainability, and health risks. Insight from Abruzzo, Italy. J. Food Compos. Anal. *114*, 104797 https://doi.org/10.1016/j.jfca.2022.104797.

Shami-shah, A., Travis, B.G., Walt, D.R., 2023. Advances in extracellular vesicle isolation methods: A path towards cell-type specific EV isolation. Extracell. Vesicles Circ. Nucleic Acids *4* (3), 447–460. https://doi.org/10.20517/evcna.2023.14.

Shinge, S. a U., Xiao, Y., Xia, J., Liang, Y., Duan, L., 2022. New insights of engineering plant exosome-like nanovesicles as a nanoplatform for therapeutics and drug delivery. Extracell. Vesicles Circ. Nucleic Acids *3* (2), 150–162. https://doi.org/10.20517/evcna.2021.25.

Street, J.M., Koritzinsky, E.H., Glispie, D.M., Yuen, P.S.T., 2017. Urine exosome isolation and characterization. In: Gautier (A c. Di), J.-C. (Ed.), Drug Safety Evaluation: Methods and Protocols. Springer, pp. 413–423. https://doi.org/10.1007/978-1-4939-7172-5_23.

Subha, D., AnuKiruthika, R., Sreeraj, H., Tamilselvi, K.S., 2023. Plant exosomes: nano conveyors of pathogen resistance. Discov. Nano *18* (1), 146. https://doi.org/10.1186/s11671-023-03931-4.

Sun, D., Zhao, Z., Spiegel, S., Liu, Y., Fan, J., Amrollahi, P., Hu, J., Lyon, C.J., Wan, M., Hu, T.Y., 2021. Dye-free spectrophotometric measurement of nucleic acid-to-protein ratio for cell-selective extracellular vesicle discrimination. Biosens. Bioelectron. *179*, 113058 https://doi.org/10.1016/j.bios.2021.113058.

Taylor, H., Webster, K., Gray, A.R., Tey, S.L., Chisholm, A., Bailey, K., Kumari, S., Brown, R.C., 2018. The effects of «activating» almonds on consumer acceptance and gastrointestinal tolerance. Eur. J. Nutr. *57* (8), 2771–2783. https://doi.org/10.1007/s00394-017-1543-7.

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G.K., Ayre, D.C., Bach, J.-M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N.N., Baxter, A.A., Bebawy, M., Zuba-Surma, E.K., 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles *7* (1), 1535750. https://doi.org/10.1080/20013078.2018.1535750.

U.S. Department of Agriculture, *A.R.S. U.S. Department of Agriculture, Agricultural Research Service. FoodData Central. 2022*. Available online: Https://fdc.nal.usda.gov/ (accessed on 20 march 2023).

Waghmare, S., Lileikyte, E., Karnik, R., Goodman, J.K., Blatt, M.R., Jones, A.M.E., 2018. SNAREs SYP121 and SYP122 mediate the secretion of distinct cargo subsets. Plant Physiol. *178* (4), 1679–1688. https://doi.org/10.1104/pp.18.00832.

Yakubovich, E.I., Polischouk, A.G., Evtushenko, V.I., 2022. Principles and problems of exosome isolation from biological fluids. Biochem. (Mosc.), Suppl. Ser. A: Membr. Cell Biol. *16* (2), 115–126. https://doi.org/10.1134/S1990747822030096.

Yang, C., Zhang, M., Merlin, D., 2018. Advances in plant-derived edible nanoparticle-based lipid nano-drug delivery systems as therapeutic nanomedicines. J. Mater. Chem. B *6* (9), 1312–1321. https://doi.org/10.1039/C7TB03207B.