Using an innovative size-exclusion extraction system aimed at the isolation of plant-derived exosomes

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Abstract. Exosomes are nanosized extracellular vesicles abundantly found in biofluids and plant biomass, capable of transmitting biological signals and participating in intercellular communication, immune regulation, tissue regeneration, and tumor development. The scientific community's strong interest in the biological effects of exosomes and their various clinical, nutraceutical, and cosmetic applications has driven the search for efficient, simple, and cost-effective methods for isolating and purifying exosomes.
This work introduces an innovative extraction system using a refrigerant solvent in sub-critical conditions that could serve as a straightforward, effective, and scalable alternative to traditional methods currently used for isolating exosomes from both human and plant sources. Extraction trials were conducted on wet and partially dried broccoli sprouts using a lab-scale, semi-automated plant equipped with an on-site homogenizing system to promote disruption of the solid cellular structure and the availability of exosomes in the plant matrix; moreover, a suitable filtration unit was investigated to obtain their size-based separation from the extract.
Although the initial setup included two polycarbonate filters in sequence with decreasing mesh size downstream of the extraction chamber, using a single stainless steel filter and recovering the extract in a preserving phosphate-buffered saline solution proved to be the best approach in terms of process feasibility and recovery of intact, non-aggregated vesicles.

1. Introduction

Recently, exosomes have attracted growing interest in applied life sciences and biotechnology research.
They are extracellular vesicles produced by eukaryotic cells and originate from endosomes, with diameters ranging from 50 nm to 200 nm [1, 2].
They play an important role in intercellular signaling by facilitating the transfer of bioactive compounds, thereby regulating both normal physiological processes and various pathological conditions [3].
In particular, plant-derived exosomes can serve as a natural nanodelivery system, offering advantages such as greater immune tolerance, diversity of origin, and safety compared to animal-derived exosomes [4].
The main plant-based matrices with high exosome concentrations include ginger, grapefruit, broccoli, pomegranate, apple, and orange [5].
Current techniques for exosome recovery include ultracentrifugation, ultrafiltration, precipitation, immunoaffinity capture, and size-exclusion chromatography [3]. However, these methods are difficult to implement industrially due to sample loss, low isolation efficiency, low exosome recovery and purity, and extremely high costs, highlighting the need for innovative solutions [6].
This research project aimed to demonstrate the feasibility of isolating exosomes from moist and partially dried broccoli sprouts using a novel semi-automated extraction apparatus that employs subcritical fluid R1234ze, a solvent whose potential has already been demonstrated for the recovery of lipophilic substances from natural matrices [7].
However, in the present work, it was proposed as a drag fluid, without dissolving the exosomes and maintaining their lipophilic membrane intact by installing a homogenization system, thereby ensuring an adequate turbulence regime in the extraction chamber.
Additionally, the implementation of an appropriate filtration system downstream of the extraction chamber was examined to enhance extract purification and minimize exosome aggregation.
Extracts were characterized for sample size heterogeneity, vesicle membrane integrity, and exosome identity by confocal fluorescence microscopy, followed by Western blot analysis to evaluate the expression of Tetraspanin 8 (TET-8), a specific marker of plant-derived exosomes.

2. Materials and Methods

2.1. Raw materials

Wet broccoli sprouts (*Brassica oleracea*) were cultivated in vitro and provided by Arterra Bioscience SpA for experimentation (initial moisture content, 90% w/w). Before extraction, coarse grinding was performed using a commercial miller; in addition, partially dried samples were obtained by incubating the samples at 40°C

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in an oven for 24 h to reach a final moisture content of 30% w/w.

The refrigerant R-1234ze Solstice® (purity grade > 99.8 %) used for extractions was purchased from General Gas (Milan, Italy).

A purified exosome extract (10^{10} particles per mL) was provided by the Research Group belonging to the Biology Department, University of Tor Vergata (Rome) and used as a positive control for analyses.

2.2. Extraction system and experimental trials

The lab-scale extractor used for experiments is based on a patented process [8], described by Colucci Cante et al. [7], with some configurational adaptations.

The liquid percolates through the solid matrix, previously loaded into an extraction chamber and appropriately homogenized through an in-line Ultra Turrax system.

After purification through a filtration unit, it reaches a jacketed expansion chamber; here, it evaporates due to depressurization while the extract is released to the bottom, is sucked by a compressor, and is recondensed and recirculated, being fully regenerated.

Table 1 presents the system configurations and operating conditions used for the extraction tests.

Table 1. Extraction trials (T) performed on Broccoli sprouts using the solvent R1234ze.
PC: Polycarbonate; PBS: phosphate buffer saline; EP: extraction pressure; VP: vaporization pressure.

#T	*Filters*	*Collection fluid*	*Time (min)*	*Matrix*	*EP* (bar)	*VP (bar)*
#1	PC: 200 nm PC: 30 nm	-	30	wet	6	3
#2	PC: 200 nm PC: 30 nm	-	30	semi-wet	6	3
#3	PC: 200 nm	PBS	30	semi-wet	6	3
#4	No filter	PBS	30	semi-wet	6	3
#5	Stainless steel	PBS	30	semi-wet	6	3

2.3. Analytical methods

Extracts from each extraction run were collected using 4 mL of PBS (10 mM, pH 7.2), taking care to recover all residues from the walls of the expansion chamber.

Therefore, all samples were analysed both without any pretreatment (fresh samples, F) and after centrifugation at 50000 rpm for 2 h using a Beckman Optima L-90K ultracentrifuge (Beckman Coulter) equipped with an SW55 rotor.

The resulting pellets were resuspended in 100 µL PBS and stored at -20°C (ultracentrifuged samples, UC).

To assess extracellular vesicle properties, a multi-analytical approach was applied.

After a preliminary Dynamic Light Scattering (DLS) analysis to determine particle size distribution and sample heterogeneity, PKH26 staining combined with confocal microscopy was used to evaluate vesicle membrane integrity and their visualization.

Finally, a Western Blot (WB) of TET8, a plant-derived extracellular vesicle-associated tetraspanin involved in vesicle formation and membrane organization, was performed to confirm vesicle identity.

For confocal microscopy, samples were stained with PKH26 (Sigma-Aldrich), a lipophilic carbocyanine dye that intercalates into the lipid bilayer of extracellular vesicles, enabling stable membrane labeling without altering vesicle structure.

PKH26 emits in the red spectrum (excitation ~551 nm, emission ~567 nm) and was detected using a red fluorescence channel. Imaging was performed using a ZEISS LSM 900 confocal microscope with a 63× objective. Subsequently, the protein content of all preparations was determined using the Bradford assay [9], and Western blot (WB) analysis was performed to evaluate TET8 protein expression [10].

WB was performed using 10% SDS-PAGE to detect a ~ 31 kDa protein. Membranes were incubated with anti-plant Tetraspanin 8 antibody (TET8; PhytoAB, Cat. No. PHY1490A, Lot 094102).

Up to 35 µL of sample was loaded per well due to low protein concentration. Signal was detected using ECL ONE STAR (EuroClone, Cat. No. EMP003250) and visualized with a ChemiDoc MP Imaging System (Bio-Rad).

3. Results and Discussion

Trials #1 and #2 were initially conducted on wet and semi-dried broccoli, respectively, using a double-layer polycarbonate filtration system (0.2 – 0.03 µm mesh size) to recover exosome-rich extracts from the smaller filter. This setup revealed a filter failure due to excessive packing of the wet matrix and/or high pressure drops across the 0.03 µm filter, resulting in slow filtration and difficulty in operating the equipment at full capacity.

Since the nonpolar refrigerant could compromise vesicle integrity due to its affinity for the exosomes' double lipid membrane, the presence of residual water and turbulence within the extraction chamber was necessary to ensure adequate dispersion and entrainment of particles by the extracting fluid, without achieving effective dissolution. Therefore, it was necessary to redesign the filter housing to increase the filter surface area while reducing pressure drops. As a result, double filters were replaced with a single 0.2 µm filter (in PC and stainless steel for trials #3 and #5, respectively), and the exosome-rich extracts were collected in PBS to prevent particle aggregation [11].

DLS results revealed partially overlapping size distributions for stainless steel filter samples, with detectable particle populations in the 50 – 250 nm range and acceptable correlation profiles.

In contrast, polymer-filter and no-filter samples exhibited broad, poorly defined distributions, dominated by large particles (>1 µm) and high polydispersity, indicating aggregation and poor sample quality, with minimal representation of nanoscale vesicles.

As shown in Figure 1, confocal fluorescence microscopy revealed the presence of large aggregates in extracts from trial #3 (panels A and B) and in fresh samples from trials

#4 (panel C); smaller aggregates were also visible in ultracentrifuged samples from #4 (panel D).
In contrast, the absence of aggregates and the visualization of distinct particles is evident in Panels E and F (trial #5).

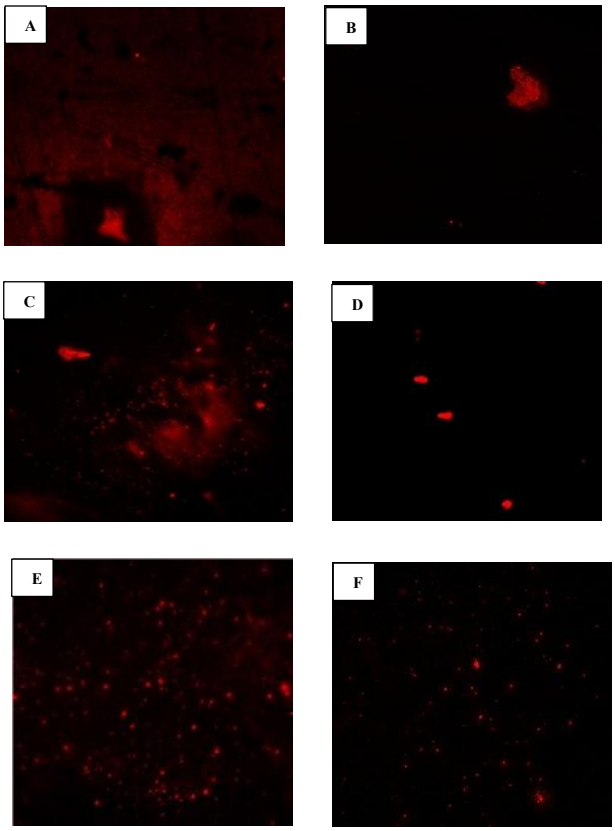

Figure 1: Immunofluorescence images. A: fresh polymer filter extract; B: ultracentrifuged polymer filter extract; C: fresh no-filter extract; D: ultracentrifuged no-filter extract; E: fresh stainless steel filter extract; F: ultracentrifuged stainless steel filter extract.

Western blot analysis targeting TET8 confirmed these findings, as observed in Figure 2.
Polymer-filter and no-filter samples exhibited a pronounced nonspecific fluorescence and substantial aggregation, which hindered vesicle detection.
In contrast, the use of a stainless filter facilitated the identification of vesicles with defined morphology and minimal background, as represented in Panels E and F.

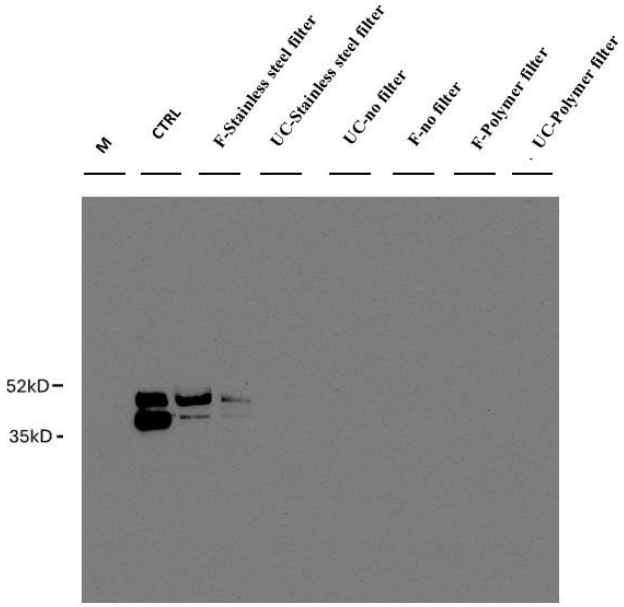

Figure 2: Western blot results. M: marker; CTRL: purified exosome extract used as a positive control; *F: Stainless steel filter*: fresh extract using a stainless steel filter; *UC-Stainless steel filter*: ultracentrifuged extract using a stainless steel filter; *UC-no filter*: ultracentrifuged extract without using a filter; *F-no filter:* fresh extract without using a filter; *F-Polymer filter*: fresh extract using a polymer filter; *UC-Polymer filter:* ultracentrifuged extract using a polymer filter.

A specific TET8 signal was detected in the positive control and in stainless steel filter samples, both fresh and ultracentrifuged, while polymer filter and no-filter samples showed no detectable signals.
In particular, the TET8 band detected in the F-stainless steel filter sample was slightly more intense than that observed in the UC extract prepared with a stainless steel filter, suggesting possible vesicle damage during ultracentrifugation.
Overall, these results indicated that polymer-based filtration likely retains vesicles within aggregates, preventing their detection across all analytical techniques.
The exosome retention on the polycarbonate membrane, compared to the steel filter, could be attributable to the hydrophobic/aromatic interactions between the polymeric phenyl groups and the galactolipids present on their membrane [12].
In no-filter samples, vesicles were not physically retained but were highly diluted within the total recovered material, limiting their detectability.
In contrast, the stainless-steel filter enabled efficient recovery of vesicles, preserving their integrity and allowing consistent detection across DLS, confocal microscopy, and Western blot analysis.

4. Conclusions

This study demonstrates the potential of an innovative extraction system that utilizes a refrigerant solvent under sub-critical conditions as a straightforward, effective, and

scalable alternative to conventional methods for isolating exosomes from plant sources such as broccoli.

The system included an integrated homogenizing unit for processing the solid matrix and a filtration module designed for size-based separation of the extracts.

Using a stainless-steel filter yielded exosome preparations comparable to those obtained by the classical ultracentrifugation method (positive control).

This approach preserved material integrity, reduced nonspecific adhesion, and minimized background contamination, as demonstrated by confocal microscopy.

Western blot analysis of TET8 protein further confirmed the identity of the vesicles present in extracts from the stainless-steel filter configuration. Future studies will focus on optimizing exosome recovery and purification by refining the filtration unit to improve size-exclusion efficiency.

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