Application note

Isolation of extracellular vesicles from *Citrus limon* juice using Phoebus SEC Column

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Introduction

Extracellular vesicles (EVs) are spherical, membrane-bound structures secreted by living cells.[1] Intercellular communication is considered to be their dominant function. The composition of the vesicle reflects the phenotype of the secreting cell, which can be used for disease diagnosis.[2] EVs are also recognized as a novel class of biopharmaceutics and drug carriers.[3]

Plant material is an important alternative source of EVs.[4] It's relatively cheap, readily available, and abundant in EVs.[5] The *Citrus limon* fruit-derived EVs are one of the most exhaustively studied. The vesicles isolated from lemon juice were shown to carry biomacromolecules (like proteins, nucleic acids, and lipids) and metabolites (flavonoids and triterpenes).[6] They were shown to exhibit anti-inflammatory,[6,7] antioxidant,[7] and tumor-inhibitory properties.[8–10]

The technical note demonstrates the methodology for the isolation of small EVs from *Citrus limon* fruit (lemon) juice samples using a size-exclusion chromatography (SEC) column: Phoebus SEC Column. The column was designed for the rapid purification of EVs from plant material and bacterial culturing media with high recovery.

The methodology includes differential centrifugation and filtration of the plant material for the removal of particulate matter, initial sample preconcentration with ultrafiltration (UF), and retentate purification using a Phoebus SEC Column. The obtained isolates were characterized in terms of total protein content, particle size distribution, and particle concentration. The purity of EVs was assessed with the particle-to-protein ratio and the capillary electrophoresis technique. The identity of the isolated structures was confirmed with electron microscopy.

Methods

Materials: All chemicals used in the experiments were of analytical grade or higher. Phosphate buffered saline (PBS; prod. id. P4417) tablets, bovine serum albumin (BSA; prod. id. A9647), sodium dodecyl sulfate (SDS; prod. id. 75746-250G), and disodium tetraborate decahydrate (borax; prod. id. S9640) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was obtained from Avantor (prod. id 810981118; Gliwice, Poland).

EV isolation protocol: Lemons were purchased in a local grocery shop. Fruits were washed with lukewarm water and mechanically squeezed. The juice was transferred into 50 mL conical centrifuge tubes (prod. id. 11312202; Hawach Scientific, Xi'an, China) and was centrifuged at 4,000 RCF (20 °C) for 30 min in an Allegra X-22R centrifuge (Beckman, Brea, CA, USA). The supernatant was collected and filtered through a 5.0 µm cellulose acetate syringe filter (prod. id. 11058228; Hawach Scientific). The filtrate was centrifuged at 10,000 RCF (20 °C) for 30 min. The supernatant was collected and centrifuged at 20,000 RCF (20 °C) for 30 min. The liquid was carefully collected and filtered through a 0.22 µm polyethersulfone (PES) syringe filter (prod. id. 11048405; Hawach Scientific). 10 mL of the filtrate was transferred to Vivaspin 20 (prod. id. VS2052; 300 kDa; PES filter; Sartorius; Göttingen, Germany) concentrator and centrifuged until the retentate volume was reduced to 0.5 mL. The retentate was fractionated with a Phoebus SEC Column (EVS-SEC-001; EV Scientific) using PBS solution as an eluent. Ten fractions were collected (0.5 mL each). Fractions 4 – 6 were combined and preconcentrated with Amicon Ultra 0.5 (50 kDa;

regenerated cellulose membrane; 0.5 mL; Merck) concentrator at 3,000 RCF until the volume was reduced to 200 µL. The isolate was characterized according to the International Society of Extracellular Vesicles recommendations.[11]

Bicinchoninic acid assay (BCA): BCA was performed according to the vendor recommendations (QPRO-BCA Kit Standard, Cyanagen, Bologna, Italy). Samples and standard solutions were mixed with 6% SDS solution in a 9:1 volumetric ratio. A calibration curve was constructed in a range of 0 – 2000 µg mL^{-1} using BSA stock solution in PBS. The measurements were performed with a DTX-880 plate reader (Beckman) at 562 nm wavelength.

Tunable resistive pulse sensing (TRPS): The measurements were performed with the Exoid system (IZON, Christchurch; New Zealand) using a three-point calibration method. The NP150 nanopore and CPC100 calibration standards were used. The desired sensitivity was obtained with a stretch of 46 mm and a pressure of 300 – 900 Pa. The voltage of 1000 mV was applied to adjust the baseline current to 120 ± 10 nA.

Capillary electrophoresis: The electrophoresis was conducted with a PACE MDQ system (Beckman) equipped with a photodiode array detector. Bare fused silica capillaries (50 µm i.d. x 363 µm o.d. x 30.2 cm of total length; Beckman) were used. The background electrolyte was composed of 25 mM borate buffer (pH 9.2). The sample was injected hydrodynamically (5 s, 3.45 kPa) and separated under constant voltage (10 kV) conditions. The analysis was monitored at 200 nm. Before each analysis, the capillary was conditioned with 0.1 M NaOH (1 min), water (1 min), and background electrolyte (2 min) using 137.9 kPa pressure.

Cryogenic electron microscopy (cryo-TEM): 3 µL of the sample was blotted onto a discharged lacey formvar/silicon monoxide 300 mesh copper grid (Ted Pella Inc., Redding, CA). A blotting force of 2 was applied for 3 s at 4 °C using a Vitrobot Mark IV (Thermo Fisher, Waltham, MA). Liquid ethane was used for vitrification. Images were captured using a FEI Tecnai G2 20 TWIN transmission electron microscope (Hillsboro, OR). The microscope was operated at a voltage of 200 kV in a low-dose setup, and a FEI high-sensitive Eagle camera.

Results and discussion

SEC fractions characterization: The fractions obtained with the Phoebus SEC Column were characterized with BCA and TRPS (Figure 1). The protein and particle concentration remained negligible in the first three fractions (void volume). Fractions 4 – 6 were abundant in proteins and particles, which indicated EV-rich fractions. A gradual decrease in particle concentration was observed in fractions 5 – 8. Protein content remained low in fractions 7 and 8 but significantly increased in the last two fractions (9 and 10). Fractions 9 and 10 were also devoid of detectable concentrations of particles. According to the particle concentration measurements, fractions 4 – 6 contained more than 93.1% of the particles detected in all fractions.

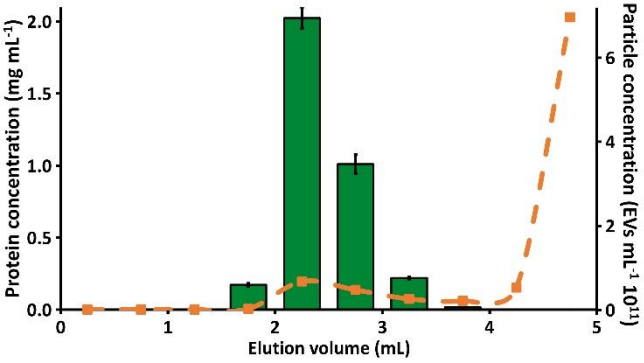

Figure 1. The elution profile of the *C. limon* fruit juice sample fractionated with the Phoebus SEC Column. The orange trace represents protein concentration (BCA), and the green bars demonstrate particle concentration (TRPS).

Capillary electrophoresis confirmed the presence of EVs in fractions 4 – 8 (Figure 2). The analyses demonstrated high purity of fractions 4 – 6. Although EVs were observed in fractions 7 and 8, numerous low-intense signals were also detected, indicating deterioration of the fractions' purity. In turn, fractions 9 and 10 were shown to be a complex mixture of various components. It should be emphasized that none of these signals were observed in EV-rich fractions.

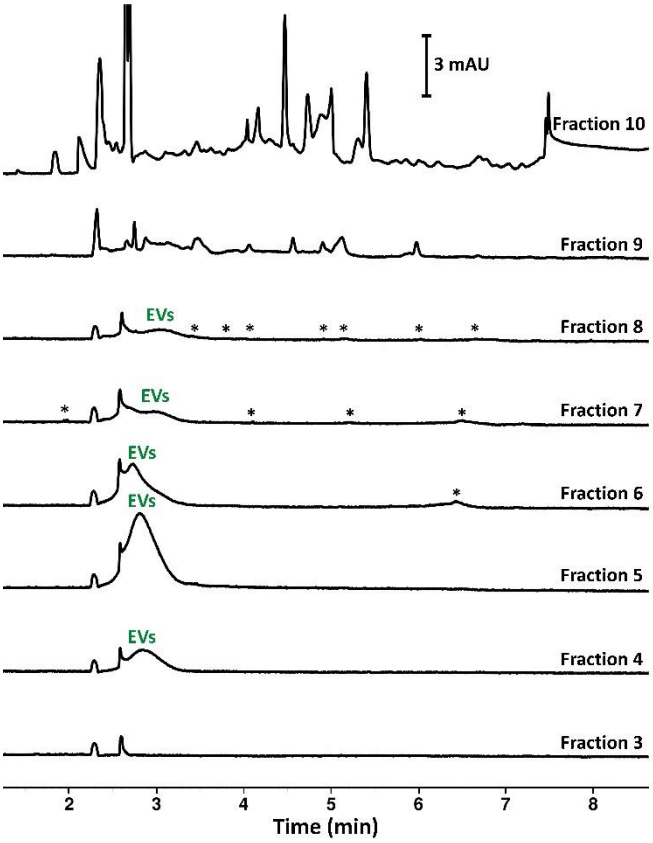

Figure 2. The capillary electrophoresis analyses of fractions obtained during the fractionation of the *C. limon* fruit juice sample with the Phoebus SEC Column. Abbreviations: EVs – extracellular vesicles; * – asterisks indicate low-intensity peaks (impurities).

Final isolate characterization: Fractions 4 – 6 were combined and preconcentrated down to about 200 µL using an Amicon Ultra 0.5 concentrator. More than 99% of vesicles were in the size range of 60 – 160 nm (Figure 3). The mean, mode, and median size (± standard deviation) were equal to 81 ± 3 nm, 68 ± 3 nm, and 73 ± 4 nm (two independent experiments), respectively. These observations are in good agreement with the particle size distribution reported for the plant exosome-like vesicles.[6-10]

In total, about 120 µg of EVs (based on protein content) were obtained, which corresponded to 2.5 * 10^{11} particles. The particle-to-protein ratio of the isolate was 2.1 * 10^{9} ± 1.4 * 10^{7} particles μg^{-1}.

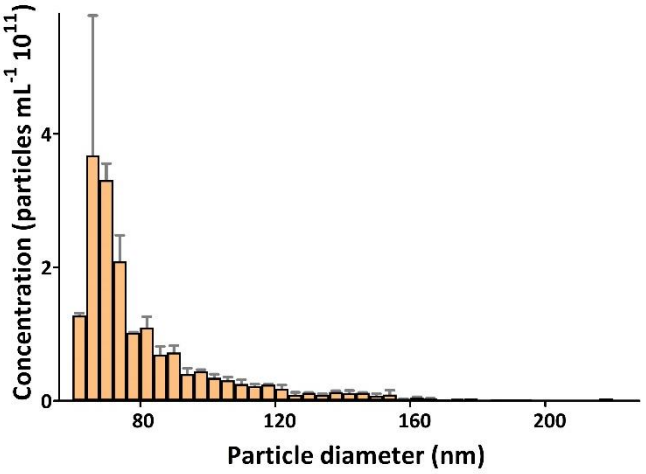

Figure 3. The size distribution of EVs isolated from the *C. limon* fruit juice samples. The error bar represents a standard deviation of the mean (three TRPS measurements).

Electron microscopy confirmed the presence of round-shaped vesicular structures (Figure 4). The size of the observed objects was within the scope determined by TRPS measurements (Figure 3). Moreover, the phospholipid membrane was clearly visible, which confirmed the identity of EVs.

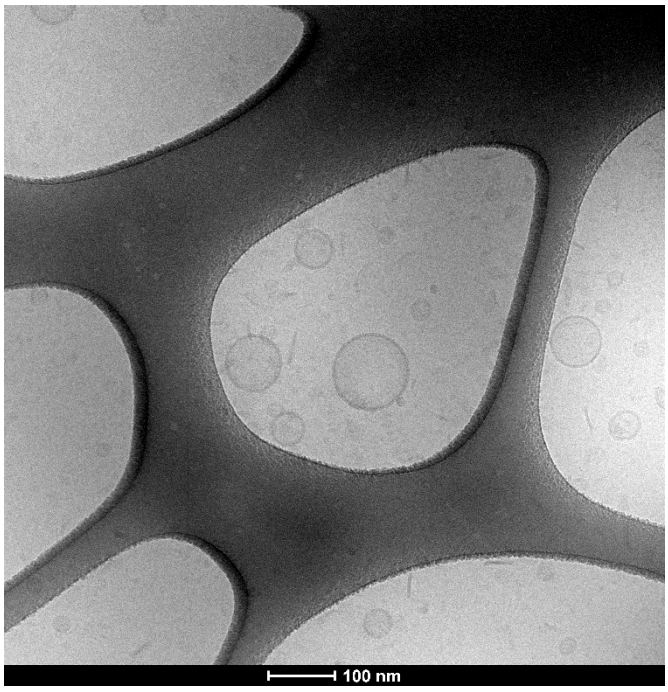

Figure 4. The cryo-TEM image of the EVs isolated from the *C. limon* fruit juice samples.

The purity of the isolate was assessed with capillary electrophoresis (Figure 5). A single low-intense signal was observed next to the main EV peak. The relative content of EVs in the isolate was >98.5%.

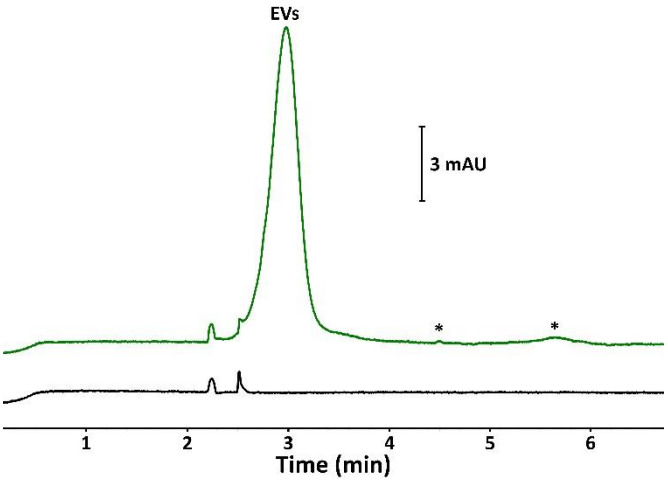

Figure 5. The capillary electrophoresis analysis of the final isolate. Abbreviations: EVs – extracellular vesicles; * – asterisks indicate low-intensity peaks (impurities).

Summary

A demonstrated methodology enables the isolation of small (<160 nm) extracellular vesicles from *Citrus limon* juice samples. The application of the Phoebus SEC Column was essential for the removal of sample matrix components. The implementation of the presented protocol enables rapid purification of EVs with high recovery (>93.1%). The quality of the finally obtained isolates was confirmed with particle-to-protein ratio and capillary electrophoresis. The estimated purity of the isolate exceeded 98.5%.

References

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Ordering information

Product name	Product ID	Supplier
Phoebus SEC Column	EVS-SEC-001	EV Scientific
50 mL conical centrifuge tubes	11312202	Hawach Scientific
5.0 µm cellulose acetate syringe filter	11058228	Hawach Scientific
0.22 µm PES syringe filter	11048405	Hawach Scientific

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