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“Excellence hub in green technologies: Introducing innovation ecosystems in the Mediterranean food value chain”

EXCEL4MED

Report on green extraction process of pomegranate and citrus seed oil for improved fatty acid and bioactive profile

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Abbreviation

BACs — bioactive compounds

CD — convective drying

CE — catechin equivalents

FD — freeze drying

GAE — gallic acid equivalents

GETs —Green Extraction Techniques

HCAE — hydrodynamic cavitation-assisted extraction

MD — microwave drying

PEF — pulsed electric fields

PS — pomegranate seed extract

PSO — pomegranate seed oil

PUFAs — polyunsaturated fatty acids

RSM — response surface methodology

s:s — solvent-to-seed ratio

TE — Trolox equivalents

TFC — Total Flavonoid Content

TPC — Total Phenolic Content

TTC — Total Tannin Content

UAE — ultrasound-assisted extraction (using both bath and probe configurations)

Abstract

This report presents the development, optimization, and evaluation of green extraction technologies for the recovery of bioactive compounds and oil from pomegranate and citrus (orange) seeds, agro-industrial by-products of high environmental impact. Three green extraction techniques were employed: ultrasound-assisted extraction using both bath and probe configurations, hydrodynamic cavitation-assisted extraction, and pulsed electric fields. The study focused on optimizing key parameters independently for each extraction technique, such as solvent composition, extraction time, amplitude, and electric field strength, using response surface methodologies (e.g. Box–Behnken designs) to maximize oil yield and the recovery of phenolics, flavonoids, and tannins. Optimization was initially performed using pomegranate seeds as the model matrix. Under optimal UAE conditions, oil yields reached up to 27.35 ± 0.95%, with significantly enhanced recovery of total phenolic content (up to 11.85 ± 1.87 mg GAE/g oil). Hydrodynamic cavitation-assisted extraction and pulsed electric fields, while yielding lower extract quantities, demonstrated potential in preserving antioxidant capacity with minimal solvent use and thermal input. The same optimized parameters were later applied to citrus seeds to assess transferability and performance across matrices. Phytochemical screening revealed that ultrasound-based methods were generally more effective in recovering bioactive compounds compared to hydrodynamic cavitation-assisted extraction and pulsed electric fields. This work highlights the potential of green, energy-efficient extraction methods to valorize fruit seed by-products. These findings contribute to the EXCEL4MED objective of developing sustainable, scalable bioprocesses in the Mediterranean food value chain, supporting both environmental protection and high-value product development.

1. Introduction

Due to the industrialization of the agricultural and food industry, a major waste disposal issue has emerged (Gil & Tuberoso, 2021). Annually, around 55 million metric tons of food waste are generated, with 16.5–20.5 million tons originating from the fruit and vegetable sector (Saini et al., 2019). About 30–50% of this waste is in the form of agricultural by-products, creating both economic and ecological challenges, including an estimated 8% contribution to greenhouse gas emissions (Gil & Tuberoso, 2021) . Valorizing such waste can benefit both the environment and human health (More et al., 2022), as fruit residues are rich in bioactive compounds (BACs) (Azmir et al., 2013; Jha & Sit, 2022). Along with primary metabolites like proteins, lipids, and carbohydrates, plants produce secondary metabolites such as polyphenols, flavonoids, vitamins, essential oils, organic acids, and tannins (Sagar et al., 2018). These BACs possess antioxidant, antibacterial, and anti-inflammatory properties (Saini et al., 2019) and are widely used by the pharmaceutical, cosmetic, and food industries (Gil & Tuberoso, 2021; Manconi et al., 2016).

Conventional solid-liquid extraction techniques (e.g., distillation, Soxhlet extraction, maceration, percolation, squeezing) have long been applied for extracting BACs from fruits and vegetables (Sagar et al., 2018). However, they present several downsides, including environmental impact from solvent use, potential toxicity of solvent residues, degradation of thermolabile compounds, high energy consumption, and limited selectivity (Chemat et al., 2012; Naviglio et al., 2019; Sagar et al., 2018). To address these issues, green extraction techniques (GETs) have emerged as sustainable alternatives (Cvjetko Bubalo et al., 2018). GETs aim to provide cleaner extracts, reduce energy use, replace harmful solvents with safer ones (e.g., water or agro-solvents), and valorize agro-industrial by-products (Chemat et al., 2012; Gil & Tuberoso, 2021; More et al., 2022).

Among the most promising green extraction techniques are those that employ physical forces to enhance mass transfer and cell disruption, such as cavitation and electroporation. These approaches are gaining increasing attention due to their efficiency, scalability, and ability to operate under milder conditions compared to conventional techniques. In this context, the present work explores the potential of cavitation-assisted methods—namely ultrasound-assisted extraction (UAE) and hydrodynamic cavitation-

assisted extraction (HCAE)—as well as pulsed electric field (PEF) extraction, focusing on their mechanisms and applicability for recovering bioactive compounds from pomegranate and citrus seeds.

1.1 Cavitation Assisted Extraction methods (UAE & HCAE)

Generally, cavitation effects rely on the fact that after successive collapse of transient bubbles, several physical effects are generated, like shear forces, shock waves, microjets and turbulence. The cavitation effects include: (1) thinning of membranes and disruption of cells caused by increased mass transfer rate and enhanced solvent penetration into the cells, as temperature and pressure generate collapse events, (2) implosion of cavitating bubbles that cause agitation in microporous particles of the matrix, intense inter-particle collision and microscopic level turbulence resulting in enhanced diffusion, (3) enlargement of pores, that allow enhanced diffusion of solvent in the matrix, which leads to its hydration and swelling, (4) cell disruption associated with the generation of highly reactive free radicals. All of these aforementioned effects allow the solvent to access the internal structure of cells and release the BACs (Panda and Manickam, 2019).

1.1.1 Ultrasound-Assisted Extraction (UAE)

The principle of ultrasound-assisted extraction is based on the generation of acoustic cavitation in the medium, in which ultrasonic waves of certain frequencies (20-1000kHz) transmit into a matrix, generating bubbles that later collapse (Panda and Manickam, 2017). The two major physical phenomena that ultrasonication involves are (1) penetration and scattering into the cell wall and (2) vibratory rinsing of cell organelles due to severe cell membrane damage (More and Arya, 2021). The reason that UAE is considered a GET relies not only on the fact that reproducibility is achieved within minutes, in contrast with other conventional techniques that require a longer time to complete the extraction, but also on the fact that less consumption of solvent and energy is required (Chemat et al., 2012).

1.1.2 Hydrodynamic Cavitation Assisted Extraction (HCAE)

In the case of hydrodynamic cavitation-assisted extraction, cavitation occurs due to the pressure variations in flowing liquid that change the geometry of the constriction. A massive amount of energy, high pressure (5000 bar) and temperature (9000-10,000°C) is released for a small period of time, due to the mechanical possessions, like shear forces and

turbulence shock waves occurred by the generation of millions of minuscule voids or microjets (Arya et al., 2020). One of the main advantages of this technology is that it can be applicable at lower temperatures making it ideal for thermolabile compounds.

1.2 Electroporation extraction methods

Although PEF has been extensively used for food preservation, processing, and microbial inactivation (E.A. and Amer Eiss, 2012), its applicability in extracting BACs from fruit and vegetable matrices can also be considered. This can be achieved by modifying the voltage applied (Heldman et al., 2010). During this method, a plant tissue is placed between two electrodes, where voltage (typically 0.5-20 kV/cm) is applied via short-duration pulses. The presence of free charges of opposite polarities across the cell membranes, give the ability to them to act like capacitors with low dielectric constant, so they exhibit low natural trans membrane potential The latter increases when external electric field is applied, leading to thinning of membrane due to the electrostatic attraction between opposite particles (Kumari et al., 2018).

1.3 Rationale

Since the growing demand for sustainable solutions in the agri-food sector has highlighted the need to valorize agricultural by-products. Traditional extraction techniques, which have been widely used, often involve environmental and economic drawbacks. For this reason, this research project aimed to determine the bioactive content of these agri-food by-products by utilising green extraction technologies. Bioactive compounds from selected by-products of pomegranate and citrus fruits (e.g., mandarin and orange) were extracted using cavitation and electroporation methods. The study initially focused on optimising these technologies by adjusting parameters that contribute to both economic and environmental impact, and then assessed the potential for scaling up these methods to an industrial level. The specific objectives were summarized as follows:

- Implementation of different Green Extraction Techniques (GETs), namely,UAE, HCAE, and PEF, to compare their impact on the recovery of bioactive compounds from pomegranate and citrus fruit by-products.

- Optimization of GET extraction efficiency either through combination or by modifying key parameters (temperature, intensity, time and use of green solvents,) that influence economic and environmental outcomes.
- Characterization of various bioactive compounds (e.g., polyphenols and fatty acids,) present in pomegranate and citrus extracts using antioxidant assays (DPPH Radical Scavenging Activity (DPPH), Total Phenolic content (TPC), Total Flavonoid Content (TFC) and Total Tannin Content (TTC))
- Comparison of GETs and CETs in terms of their efficiency in extracting bioactive compounds from pomegranate and citrus by-products.

2. Materials and Methods

2.1. Raw Material Preparation

Fresh pomegranate fruits (*Punica granatum L.*) and fresh orange fruits (*Citrus simensis*) were provided by the partner of ASPIS, a juice industry located in the region of Argos, Greece. Seeds were separated from the fruits by meticulously removing the arils and pulp from pomegranates and oranges, respectively. Then, the seeds were washed carefully by rinsing under running tap water until the washing water was clear. The seeds were stored in the freezer until they were lyophilized using a freeze dryer. Finally, the dried pomegranate and citrus seeds were grinded using a pulverizer (High Speed multi-functional crusher, MODEL-750, LEJIEYIN, China). Seed powder of pomegranate and oranges was sealed in high-density polyethene zipper bags and stored at room temperature in a desiccator until further use.

2.2. Drying and Conventional Extraction of Pomegranate Seeds

Pomegranate seeds were first subjected to pre-treatment using three conventional drying methods: convective drying (CD), freeze drying (FD), and microwave drying (MD). In convective drying, seeds were dehydrated in a hot air oven under controlled temperature and airflow conditions until a stable weight was reached. For freeze drying, seeds were frozen at −40 °C and processed under vacuum in a laboratory lyophilizer. Microwave drying involves the application of controlled microwave energy in short pulses to gradually remove moisture. Following drying, all seed samples were ground using a laboratory-scale mill to obtain a homogenous powder and stored in airtight containers under dark, dry conditions.

Following drying, pomegranate seeds were subjected to oil extraction using a modified version of the official AOAC 996.06 method. This conventional technique involves two main steps: acid hydrolysis and subsequent solvent-based extraction of fatty acids. Briefly, 2 g of dried, ground pomegranate seeds were mixed with 10 mL of ethanol to prevent emulsion formation. Acid hydrolysis was then carried out by adding 15 mL of 8.3 M hydrochloric acid and heating the mixture at 75 °C for 40 minutes in a water bath (Model: BWS-12/WB 12, AGROLAB). Following hydrolysis, oil extraction was performed using a biphasic solvent system composed of 30 mL of petroleum ether and 25 mL of diethyl ether. The extraction was repeated over three cycles to maximize oil recovery. The combined solvent fractions were then concentrated by rotary evaporation (Laborota 4000 efficient, Heidolph, Germany) to remove residual solvents. The recovered pomegranate seed oil (PSO) was stored at −20 °C until further analysis. Oil yield was calculated gravimetrically using the following equation:

$$Oil\ Yield\ (\%) = \left(\frac{weight\ of\ PSO\ (g)}{weight\ of\ raw\ material\ (g)} \right) \mathrm{x}\ 100$$

2.3. Green technologies extraction procedures

The extraction procedures employing green technologies were first optimized using pomegranate seeds as the model matrix, due to their well-documented richness in bioactive compounds and oil content. Each technology, UAE, HCAE, and PEF, was systematically studied by varying key operational parameters to maximize oil yield and recover phenolic compounds. Once the optimal conditions for each method were established based on the pomegranate seed trials, these conditions were subsequently applied to citrus seeds (orange) in order to assess their efficiency and transferability across different fruit by-products. This approach allowed for a direct comparison of extraction performance between two agriculturally significant but compositionally distinct matrices.

2.3.1. Ultrasound – Assisted Extraction

UAE was carried out using two configurations: an ultrasonic bath and an ultrasonic probe, to assess the influence of different cavitation mechanisms on extraction efficiency.

Ultrasound-assisted extraction utilizing an ultrasonic bath was performed using a sonication bath (Elma S 15 H Elmasonic) operating at a frequency of 40 kHz, with an ultrasonic power input of 250 W and a total working volume of 10 L. The experimental conditions included variation in extraction time, ethanol concentration, and solvent-to-seed (s:s) ratio, as shown in Table 4. To prevent degradation of thermolabile bioactive compounds, the temperature was maintained at 38 ± 2 °C throughout all experiments. For each extraction, 0.5 g of pomegranate seed powder was mixed with a solvent (ethanol-water mixture) and transferred into a 50 mL Falcon tube. The tubes were vortexed briefly and then placed in a 250 mL beaker filled with water. The beaker was immersed in the ultrasonic bath, ensuring that the water levels inside and outside the beaker were equal to allow efficient transmission of ultrasonic waves. After the completion of extraction, the mixtures were centrifuged. The supernatant was collected, filtered, and evaporated using rotary evaporator equipment. Each extraction was carried out at a defined time and solvent condition according to an I-optimal design to evaluate extraction efficiency through the estimation of pomegranate seeds' phytochemical content.

Table 1: Factors evaluated through an I-optimal design of the UAE method.

UAE Time (min)	Ethanol Concentration (%, v/v)	Solvent-to-Seed Ratio (g/mL)
20	0%	1:10
70	50%	1:20
95	100%	1:30
120		1:50
		1:70

Ultrasound-assisted extraction utilizing an ultrasonic probe. The experimental conditions included duty cycle, amplitude and time. The optimised ethanol concentration and solvent-to-solid (s:s) ratio values obtained from ultrasonic bath experiments were utilised in this study. To prevent degradation of thermolabile bioactive compounds, the temperature was maintained at 38 ± 2 °C throughout all experiments and was monitored using a thermocouple. For each extraction, 0.25 g of pomegranate seed powder was mixed with an

ethanol-water mixture (49:51) at a solvent-to-solid (s:s) ratio of 1:57 g/mL in a jacketed flask. After the extraction was completed, the mixture was transferred into tubes, vortexed briefly, and then centrifuged. The supernatant was collected, filtered, and evaporated using rotary evaporator equipment. Each extraction was carried out at a defined time, amplitude, and duty cycle according to a Box-Behnken experimental design to evaluate extraction efficiency through the estimation of the seeds' phytochemical content.

Table 2: Factors evaluated through a Box-Behnken design of the UAE method.

UAE Time (min)	Amplitude (%)	Duty Cycle (sec)
5	0%	0.2
10	50%	0.6
15	100%	1

2.3.2 Hydrodynamic Cavitation – Assisted Extraction

Hydrodynamic cavitation-assisted extraction was carried out using a centrifugal-type cavitation system integrated into a closed-loop circulation setup. The equipment consisted of a cavitation reactor with a centrifugal rotor, designed to induce controlled pressure variation and localised cavitation zones through high-velocity fluid flow. The system was connected to a sealed reservoir containing a mixture of water and pomegranate seeds at a specific solvent-to-solid (s:s) ratio of 1:200 g/mL. The mixture was continuously circulated through the cavitation chamber with the aid of a pump, at a rate of 1.5 ml/min (**Table 3**). The key parameter of circulation time was tested at two levels, 20 and 40 minutes, to evaluate extraction efficiency through the estimation of pomegranate seeds' phytochemical content.

Table 3: Factors evaluated for the HCAE method

HCAE Time (min)	Flow Rate (ml/min)	Solvent to Solid Ratio (g/ml)
20 40	1.5	1:200

2.3.3 Pusled Electric Fields – Assisted Extraction

The EPULSUS-BM1A-12 pulse generator (3 kW, Energy Pulse System, Lisbon, Portugal) was used, capable of delivering monopolar square wave pulses (1–200 µs) with a maximum output of 12 kV, 200 A, and frequencies up to 200 Hz. The system operated in batch mode, using a treatment chamber with a 1 cm electrode gap. Samples were tempered to 20°C before treatment. Voltage was monitored via an integrated oscilloscope (Pico Technologies), while outlet temperature was measured using a thermocouple (OM-HL-EH-TC, OMEGA) placed immediately after the chamber.

A Box-Behnken Design (**Table 4**) was applied, testing 20–80 square pulses (2–22 µs pulse width) at 2–7 kV/cm. Treatment duration was calculated theoretically (number of pulses × pulse width), ranging from 80–320 µs. These key parameters were tested to evaluate extraction efficiency by estimating the phytochemical content of pomegranate seeds.

Table 4: Factors evaluated through a Box-Behnken design of the PEF techology

Electric Field Strength (kV/cm)	Pulse Width (µs)	Pulse Number (N)
2	2	20
4.5	11	50
7	22	80

2.4 Chemical Characterization Techniques

Four key assays were employed to evaluate the phytochemical content and antioxidant activity of pomegranate and citrus seeds. These included Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Tannin Content (TTC), and DPPH Radical Scavenging Activity. All analyses were conducted using spectrophotometric techniques. Prior to analysis, pomegranate and citrus seeds samples were diluted in 10 mL of a 70:30 (v/v) ethanol–water solution. The mixtures were vortexed and treated in an ultrasonic bath (Model No: BWS-12/WB 12, AGROLAB) for 15 minutes to ensure homogenization. Subsequently, the samples were centrifuged using a NEYA-8 (REMI ELEKTROTECHNIK LTD) centrifuge at 4500 rpm for 10 minutes. Supernatants were collected and stored at −20°C until further analysis.

The Total phenolic content was determined using a slightly modified Folin–Ciocalteu method. In brief, 20 µL of the prepared oil extract was mixed with 100 µL of Folin–Ciocalteu reagent and 80 µL of sodium carbonate solution (7.5% w/v) in a 96-well plate. The mixture was incubated for 2 hours at room temperature in the absence of light. Absorbance was measured in triplicate at 765 nm using a microplate reader (SPECTROstarNano, BMG LABTECH, Ortenberg, Germany). Gallic acid was used as the standard for the calibration curve, and results were expressed in mg gallic acid equivalents (GAE) per gram of oil.

TFC was assessed using a colorimetric assay based on the aluminum chloride method, as previously described (27). In a 96-well plate, 30 µL of diluted extract was mixed with 180 µL of distilled water, followed by the addition of 10 µL of 5% $NaNO_2$. After 6 minutes, 20 µL of 10% $AlCl_3$ was added. After another 6 minutes, 60 µL of 4% NaOH was introduced, and the mixture was left to react for 15 minutes. Absorbance was read at 510 nm using the same microplate reader. Catechin was used as the calibration standard, and TFC was expressed in mg catechin equivalents (CE) per gram of oil.

The total tannin content was determined following a heat-induced colorimetric method (27) using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). For each sample, 1 mL of diluted oil was combined with 0.5 mL of distilled water and 1.5 mL of 12 N HCl in two identical tubes. One tube was heated in a water bath at 100 °C for 30 minutes, while the other was kept at room temperature. Following rapid cooling, 0.25 mL of ethanol was added to both mixtures. Absorbance was measured at 520 nm, and the tannin concentration was calculated using the formula:

$$Tannin\ concentration = 19.33 * \Delta OD$$

where ΔOD represents the difference in absorbance between the heated and unheated samples, and 19.33 is a reference factor based on procyanidin oligomer content. Results were expressed as grams of total tannins per gram of oil.

Antioxidant capacity was evaluated using a slightly modified version of the DPPH radical scavenging assay. In this assay, 100 µL of the prepared oil extract was mixed with 100 µL of a freshly prepared 0.2 mM DPPH solution in 70:30 (v/v) ethanol–water. After vortexing, the mixture was incubated in the dark for 30 minutes at room temperature.

Absorbance was measured at 517 nm using the same microplate reader. Antiradical activity was expressed in mg Trolox equivalents (TE) per gram of oil using a Trolox standard calibration curve.

3. Results & Discussion

3.1. Pomegranate

3.1.1. Conventional Extraction

The results of conventional extraction demonstrated that the drying method applied to pomegranate seeds prior to extraction significantly influenced both the yield and the phytochemical properties of the resulting pomegranate seed oil extract. Among the three conventional drying techniques evaluated —freeze drying, convective drying, and microwave drying —freeze drying and convective drying produced the highest oil extract yields, with values reaching up to 15.84%. In contrast, microwave drying resulted in a noticeably lower yield of 11.93%. This reduction may be attributed to thermal stress or localized overheating, which can negatively affect cell structure and reduce solvent accessibility during extraction.

In terms of phytochemical content, the antioxidant profile of the oil extract was evaluated and the results revealed that freeze-dried samples consistently retained higher levels of antioxidant compounds. TPC values were highest in the freeze-dried oil extract, measured at 5.16 mg GAE/g oil, followed by samples derived from convectively dried seeds. Microwave-dried samples exhibited the lowest phenolic content, suggesting that elevated temperatures may have degraded heat-sensitive polyphenols. A similar trend was observed for flavonoid content, with the highest TFC recorded in the freeze-dried oil extract at 1.96 mg CE/g oil, while lower values were detected in both convective and microwave-dried samples. Tannin content also varied according to drying method, with freeze-dried and convectively dried samples presenting higher TTC values than those dried with microwaves.

The antioxidant activity of the oil extracts, assessed via the DPPH radical scavenging assay, followed the same pattern as the chromometric assays. The highest antiradical activity was observed in the oil extract obtained from freeze-dried seeds, with a value of 13.24 mg TE/g oil. Oil extracts from convective and microwave-dried seeds showed significantly lower inhibition activity, which closely paralleled the observed reductions in TPC and TFC. Figure 2 presents the antioxidant capacity of the oil extracts expressed as Trolox equivalents,

confirming that drying conditions directly influence the functional properties of the final product.

Fatty acid profiling using GC-FID revealed that all oil extracts were rich in polyunsaturated fatty acids (PUFAs), with punicic acid representing the major component in all treatments. In some cases, it accounted for up to 93.41% of the total fatty acid content. While slight variations were observed in saturated and monounsaturated fatty acids between drying methods, these differences were not statistically significant.

In summary, freeze drying proved to be the most favorable method in terms of both oil extract recovery and preservation of antioxidant compounds, without adversely affecting the lipid profile. These findings underscore the importance of pre-treatment selection in optimising both the nutritional and functional properties of pomegranate seed oil extracts for future applications.

3.1.2. Green extraction technologies

3.1.2.1. Ultrasound - Assisted Extraction via ultrasonic bath

PSO extraction was optimized using UAE under various combinations of extraction parameters, including ethanol concentration, extraction time, and solvent-to-solid ratio. A response surface methodology (RSM) with an I-optimal design was employed to evaluate these effects. Among the tested conditions, the combination of a 49:51 ethanol-to-water ratio, a 94-minute extraction time, and a 1:57 g/ml solvent-to-solid ratio yielded the highest oil extract recovery at 21.14%. (**Figure 1**) Confirmatory experiments conducted under these optimal conditions resulted in a mean yield of 21.13 ± 0.96%, validating the accuracy of the fitted second-order polynomial model. The results demonstrated the suitability of ultrasound-assisted extraction as an effective green method for recovering oil extract from pomegranate seeds. Following extraction, the chemical and functional properties of the PSO obtained under optimized UAE conditions were assessed through a series of colorimetric assays. **Table 5**, depicts the phytochemical content of PSO. The Total Phenolic Content (TPC), measured using the Folin–Ciocalteu assay, was found to be 9.0 ± 0.59 mg GAE/g oil extract. This value confirms the high phenolic density of PSO, especially when extracted under carefully optimized UAE conditions. The TFC, determined via the Aluminum Chloride method, was measured at 1.18 ±

0.32 mg CE/g oil. Tannin content, analyzed using the Total Tannin Method, was also noteworthy, reaching 6.51 ± 1.07 mg TT/g oil extract. This result is consistent with the known presence of high-molecular-weight phenolics in pomegranate by-products and reflects the

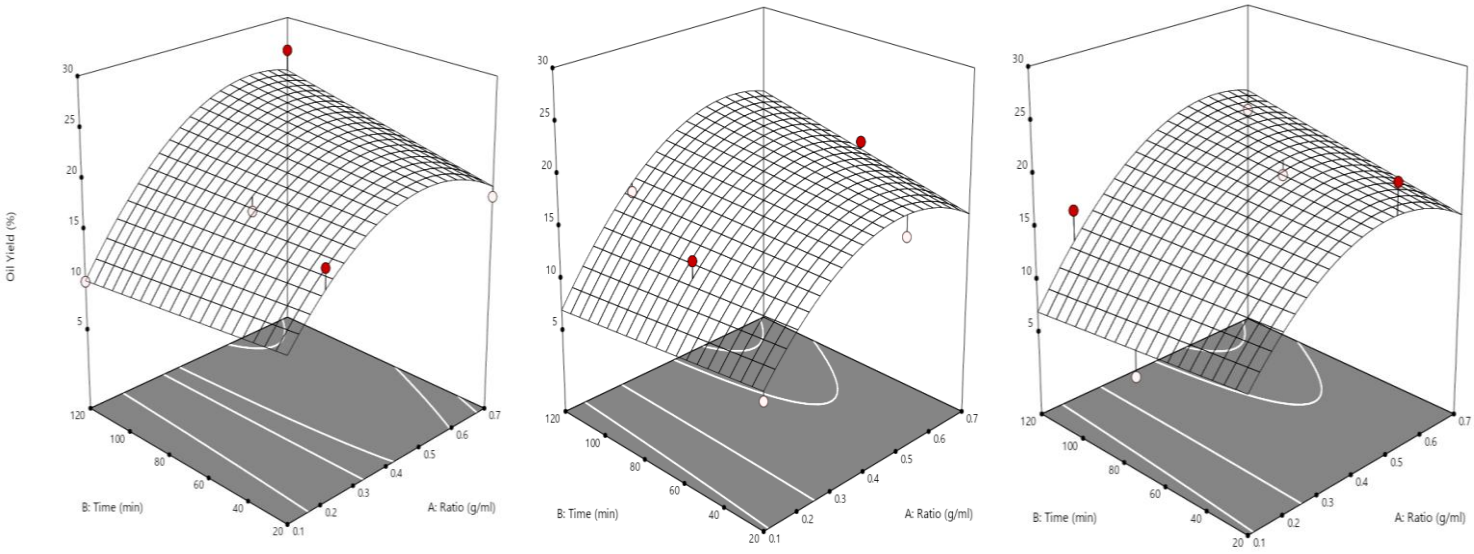

Figure 4 : 3D plots demonstrating the impact of extraction parameters (Time, Ratio and EtOH concentration) on oil extract yield. (100% EtOH Conc (left), 50% EtOH Conc (Middle), and 0% EtOH Conc (Right))

concentration of ellagitannins in the extracted oil. To assess antioxidant capacity, the DPPH radical scavenging activity assay was performed. The PSO displayed antioxidant activity of 8.53 ± 0.56 mg TE/g oil extract, demonstrating a strong antiradical potential. This result closely correlates with the high TPC and TFC values, reinforcing the conclusion that phenolic compounds are major contributors to the oil's antioxidant properties.

Table 5 : The values of TPC, TFC, TTC and Antioxidant activity in PSO extracted via ultrasound bath optimum conditions

Extraction Parameters	**TPC (mg GAE/g oil extract)**	**TFC (mg CE/g oil extract)**	**TTC (mg total tannins/g oil extract)**	**DPPH (mg TE/g oil extract)**
Optimal Conditions	9.00 ± 0.59	1.18 ± 0.32	6.51 ± 1.07	8.53 ± 0.56

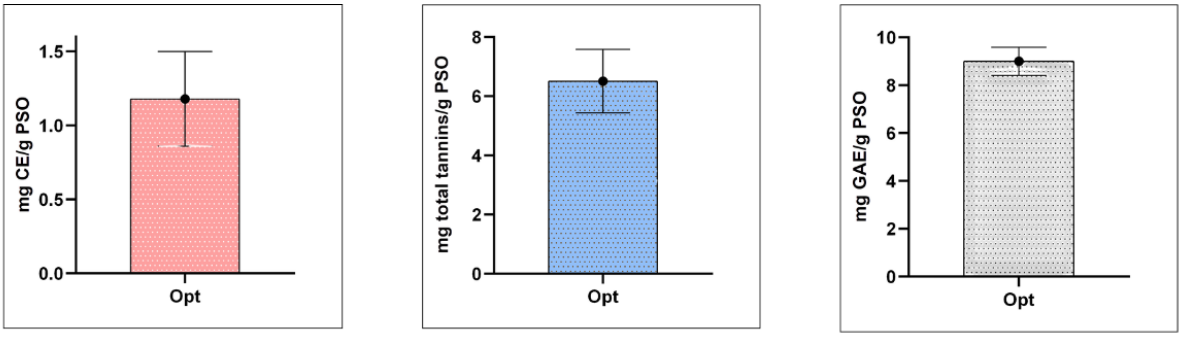

Figure 5 : Phytochemical content of PSO extracted via ultrasound bath optimal conditions (Total Flavonoid (left), Tannin (middle) and Phenolic (right)

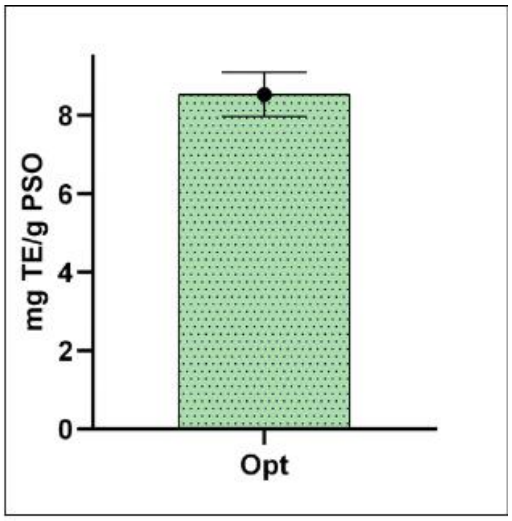

Figure 6 : Antioxidant Activity of PSO extracted via ultrasound bath optimal conditions

3.1.2.2. Ultrasound – Assisted Extraction via ultrasonic probe

PSO extraction was also investigated using UAE with a probe system, offering a more localized and intense cavitation effect compared to the ultrasonic bath. A series of experimental trials were conducted to optimize the acoustic parameters for efficient oil extract recovery. The optimal extraction conditions were identified as 60% amplitude, a duty cycle of 0.2 seconds, and an extraction time of 5 minutes. Under these conditions, the process achieved a maximum oil yield of 27.35 ± 0.95%, which surpassed the yield obtained using the ultrasonic bath. The significantly higher recovery highlights the superior intensity of energy transfer and cavitation in the probe system. Notably, the ultrasound probe achieved efficient oil extraction in just 5 minutes, compared to 94 minutes required by the ultrasound bath, demonstrating its potential as a rapid and effective green technology for the valorization of pomegranate seeds. Following extraction, the chemical and functional properties of the PSO

obtained under these optimized probe conditions were assessed through a series of colorimetric assays. **Table 6** presents the phytochemical profile of the resulting oil extract. The Total Phenolic Content (TPC), measured using the Folin–Ciocalteu assay, was found to be 11.85 ± 1.87 mg GAE/g oil extract, indicating a marked increase in phenolic compound recovery compared to bath-assisted UAE. This enhancement may be attributed to the higher localized shear forces and microstreaming generated by the probe, which facilitate more effective disruption of the seed matrix. The Total Tannin Content, determined via the Total Tannin Method, was 0.62 ± 0.07 mg TT/g oil extract. Although lower than values observed in the bath extraction, this outcome may reflect selectivity in the recovery of different phenolic subclasses under probe conditions. The TFC, analyzed using the Aluminum Chloride method, was found to be _ mg CE/g oil extract. To evaluate antioxidant potential, the DPPH radical scavenging activity was conducted, and the extract exhibited antioxidant activity of 14.20 ± 0.38 mg TE/g oil extract, indicating a strong capacity to neutralize free radicals and confirming the functional relevance of the phenolic fraction recovered under probe-assisted extraction. Together, these findings demonstrate that ultrasound probe-assisted extraction is not only time-efficient but also effective in enhancing the recovery of bioactive compounds, particularly phenolics, from pomegranate seed matrices. Its scalability and reduced processing time make it a compelling candidate for sustainable valorization of agro-industrial by-products.

Table 6 : The values of TPC, TFC, TTC and Antioxidant activity in PSO extracted via ultrasound probe optimum conditions

Extraction Parameters	**TPC (mg GAE/g oil extract)**	**TFC (mg CE/g oil extract)**	**TTC (mg total tannins/g oil)**	**DPPH (mg TE/g oil)**
Optimal Conditions	11.85 ± 1.87	10.66 ± 1.43	0.62 ± 0.07	14.20 ± 0.38

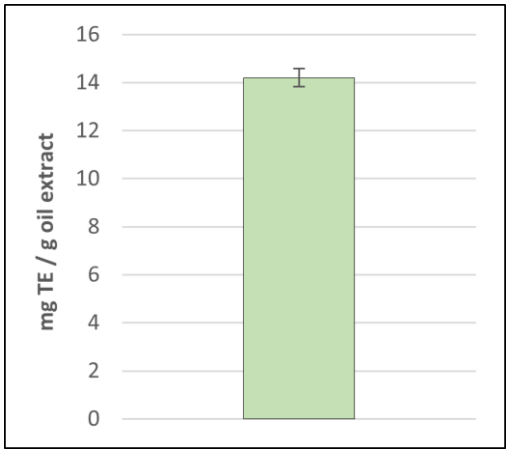

Figure 4 : Phytochemical content of PSO extracted via ultrasound probe optimal conditions (Total Flavonoid (left), Tannin (middle) and Phenolic (right)

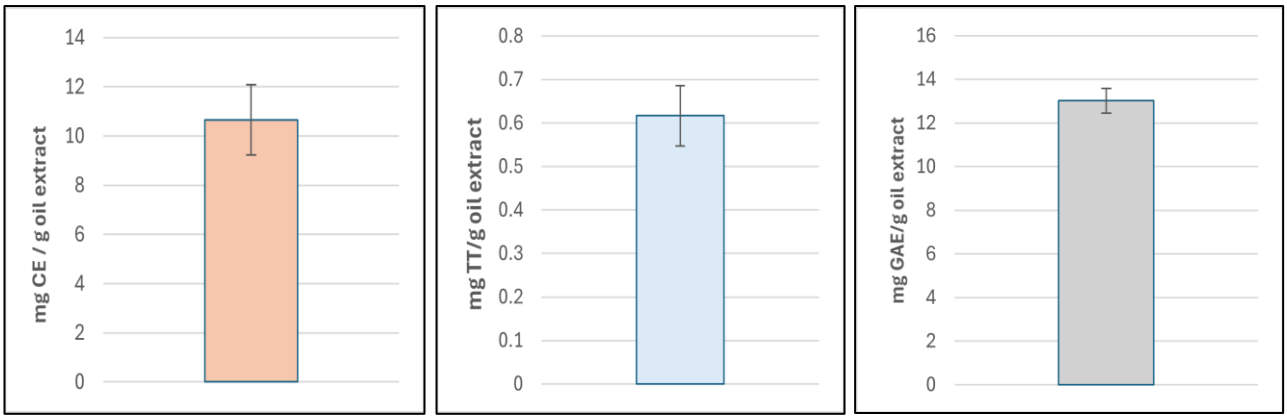

Figure 5 : Antioxidant Activity of PSO extracted via ultrasound probe optimal conditions

3.1.2.3 Hydrodynamic Cavitation - Assisted extraction

Pomegranate seed extract (PS) was obtained using a hydrodynamic cavitation-assisted extraction (HCAE) system, which leverages the collapse of cavitation bubbles to enhance mass transfer and cell disruption. Two treatment durations, 20 and 40 minutes, were investigated to evaluate the effect of processing time on extraction efficiency. The results indicated that extract yield did not differ significantly between the two durations. Similarly, the total phenolic content (TPC) remained relatively stable; however, a slight increase was observed at 40 minutes (**Figure 6**). Although not statistically significant, this trend suggests a potential for improved phenolic release with longer exposure. For this reason, the 40-minute duration was selected for all subsequent analyses (Total Flavonoid, Tannin content and DPPH Radical Scavenging Activity) to ensure the highest possible recovery of bioactive compounds without compromising process efficiency or sustainability. **Table 7** depicts the phytochemical content of PS obtained via HCAE. The TFC, determined via the Aluminum Chloride method, was measured at 3.05 ± 1.03 mg CE/g extract. Tannin content, measured at 0.39 ± 0.02 mg TT/g extract, was lower compared to both ultrasound-assisted extractions, especially the bath system, which showed notably higher tannin recovery. The DPPH radical scavenging activity assay revealed antioxidant capacity of demonstrating 13.70 ± 0.47 mg TE/g extract . This result aligns with the slightly lower phenolic and flavonoid content observed, suggesting that HCAE may not be as effective as UAE. This may be attributed to the use of a highly diluted solvent-to-solid ratio (1:200), which limits the concentration gradient and extraction efficiency, as well as to the shorter residence time and milder disruption forces compared to localized cavitation in ultrasound systems. Nevertheless, HCAE remains a clean, organic solvent-free, and scalable alternative with potential for further optimization.

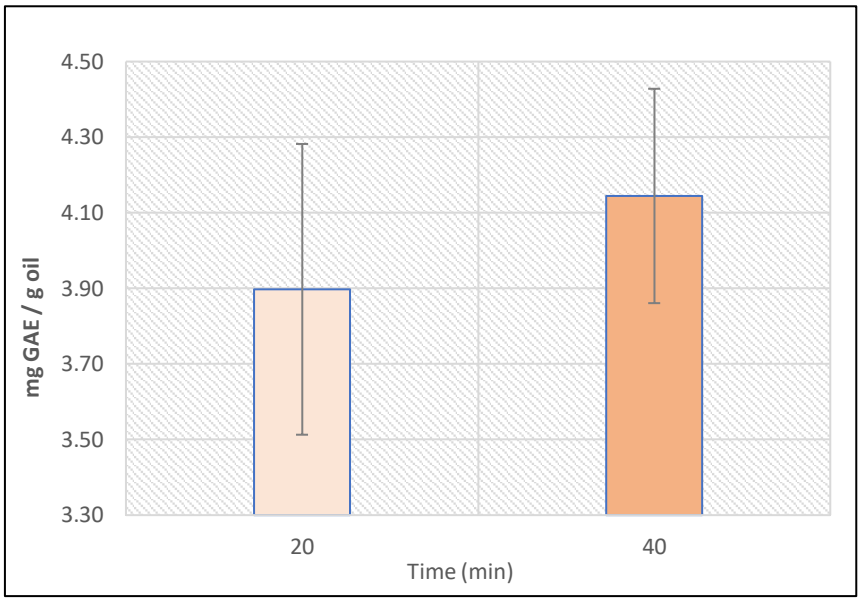

Figure 6 : Total Phenolic Content of PS obtained through Hydrodynamic Cavitation Assisted extraction

Table 7 : The values of TPC, TFC, TTC and Antioxidant activity in PS extracted via hydrodynamic cavitation optimum conditions

Extraction Parameters	**TPC (mg GAE/g extract)**	**TFC (mg CE/g extract)**	**TTC (mg total tannins/g extract)**	**DPPH (mg TE/g extract)**
Optimal Conditions	4.14 ± 0.33	3.05 ± 1.03	0.62 ± 0.07	13.70 ± 0.47

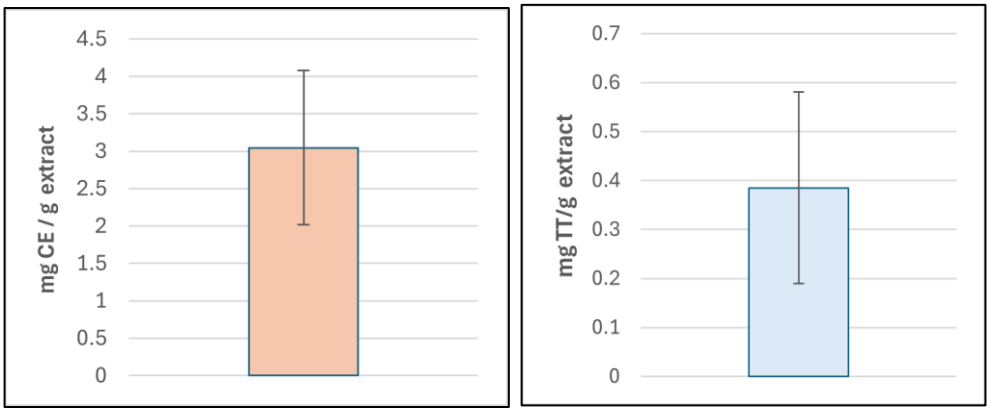

Figure 7 : Phytochemical content of PS extracted optimal conditions of hydrodynamic cavitation assisted extraction (Total Flavonoid (left), Tannin (right))

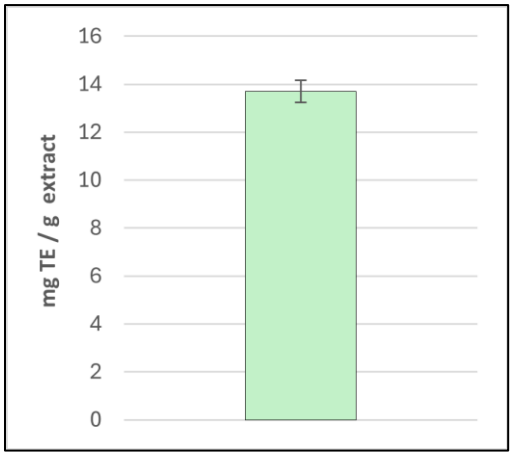

Figure 8 : Antioxidant Activity of PS extracted via hydrodynamic cavitation assisted extraction

3.1.2.4. Pulsed Electric Fields Assisted Extraction

PS was extracted obtained using PEF technology. Among the tested conditions, the combination of 80 pulses, a pulse width of 2 µs, and an electric field strength of 5.5 kV/cm was identified as optimal for maximizing cell membrane permeability while minimizing thermal effects. **Table 8** presents the chromometric assay results of PS obtained following PEF treatment. The TPC, measured using the Folin–Ciocalteu assay, was 5.04 ± 1.57 mg GAE/g extract, which is considerably lower than the values observed for both ultrasound-assisted extractions, particularly the probe system. The TFC, determined via the Aluminum Chloride method, was 6.05 ± 1.48 mg CE/g extract. The Tannin Content, measured at 1.73 ± 0.35 mg TT/g extract, was higher than obtained via HCAE, but still below the tannin content recovered through ultrasound bath extraction. The DPPH radical scavenging activity, exhibited that antioxidant capacity was at 11.92 ± 0.07 mg TE/g extract suggesting that PEF treatment was capable of releasing or activating certain phenolic antioxidants. However, both phenolic recovery and antiradical activity were generally lower compared to ultrasound-based methods. These findings suggest that although PEF alone is insufficient for efficient extraction, it holds potential as a non-thermal, pre-treatment strategy for improving the accessibility of antioxidant compounds. Notably, its application in combination with UAE could further enhance extraction efficiency and selectivity, offering a synergistic and sustainable approach for the valorization of pomegranate seed by-products.

Table 8 : The values of TPC, TFC, TTC and Antioxidant activity in PS extracted via pulsed electric field optimum conditions

Extraction Parameters	**TPC (mg GAE/g extract)**	**TFC (mg CE/g extract)**	**TTC (mg total tannins/g extract)**	**DPPH (mg TE/g extract)**
Optimal Conditions	5.04 ± 1.57	6.05 ± 1.48	1.73 ± 0.35	11.92 ± 0.07

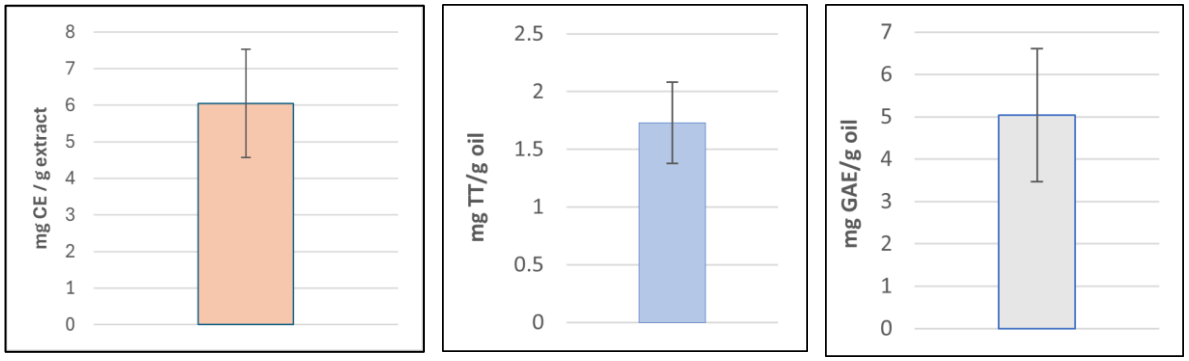

Figure 9 : Phytochemical content of PS extracted via pulsed electric fields optimal conditions (Total Flavonoid (left), Tannin (middle) and Phenolic (right)

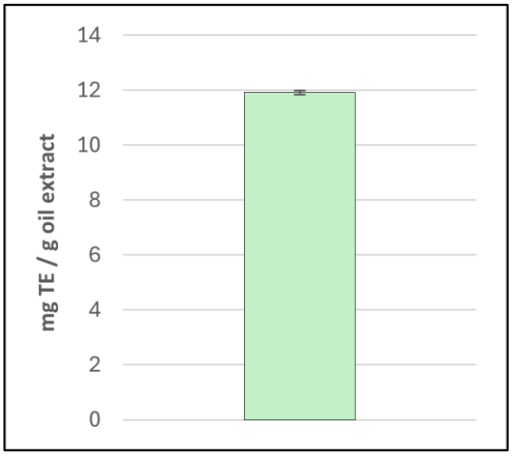

Figure 10 : Antioxidant Activity of PS extracted via pulsed electric fields optimal conditions

3.2. Orange

3.2.1. Phytochemical Screening of Orange seed oil

To assess the applicability of the optimized extraction protocols beyond pomegranate seeds, orange seeds were subjected to the same green extraction technologies—ultrasound bath, ultrasound probe, hydrodynamic cavitation, and PEF. For each method, the extraction was performed under the previously determined optimal conditions established for pomegranate seeds. Only the phytochemical profile of the resulting orange seed oil extracts was evaluated, focusing on total phenolic content, flavonoids, tannins, and antioxidant capacity. **Table 9** summarizes the phytochemical content.

Orange seed extracts obtained via ultrasound-assisted extraction using a bath system were evaluated for their phytochemical content. The TPC was measured at 7.35 ± 0.91 mg GAE/g oil extract, while the TFC was determined at 13.53 ± 1.59 mg CE/g oil extract. The Tannin Content, assessed through the Total Tannin Method, was 0.14 ± 0.01 mg TT/g oil extract. The antioxidant capacity, measured by DPPH radical scavenging activity, was calculated at 11.47 ± 0.44 mg TE/g oil extract. Compared to pomegranate seed oil extracts, the overall phenolic content and antioxidant activity in orange seed oil extracts were lower, which may be attributed to compositional differences in the seed matrix, particularly in polyphenol types and concentrations.

Ultrasound probe-assisted extraction yielded orange seed oil rich in phenolic compounds, with a TPC of 7.06 ± 1.44 mg GAE/g oil extract, TFC of 7.64 ± 0.19 mg CE/g oil extract, and tannin content measured at 0.13 ± 0.02 mg TT/g oil extract. The DPPH assay revealed an antioxidant capacity of 10.90 ± 0.62 mg TE/g oil extract. Although TFC was lower than that of the bath system, the probe system maintained strong antiradical activity. Among the methods tested, the probe system provided consistent and efficient recoveries of phenolic and flavonoid compounds, which aligns with its higher cavitational intensity and localized disruption effects.

The application of hydrodynamic cavitation to orange seeds produced a phytochemical profile characterized by a TPC of 7.49 ± 1.13 mg GAE/g oil extract, a TFC of 7.51 ± 0.49 mg CE/g oil extract, and a tannin content of 0.69 ± 0.28 mg TT/g oil extract. The DPPH assay reported a surprisingly high antioxidant capacity of 19.23 ± 1.21 mg TE/g oil

extract—higher than all other technologies. This suggests that while the total quantified phenolics were lower than in PEF-treated samples, other antioxidant compounds or synergistic effects may have contributed to the elevated radical scavenging activity.

PEF treatment of orange seed oil was conducted using a different PEF apparatus than the one employed for pomegranate seeds. The system was operated under three distinct sets of parameters, each tailored to the physical and compositional characteristics of orange seeds. Specifically, the tested conditions included: (i) an electric field strength of 6.2 kV/cm, a pulse width of 15 µs, and 5,000 pulses; (ii) 6.2 kV/cm, 15 µs pulse width, and 10,000 pulses; and (iii) 5 kV/cm, 15 µs pulse width, and 1,000 pulses. . These conditions were selected based on preliminary trials indicating that the more fibrous and dense structure of orange seed tissue required a longer energy delivery duration and a higher number of pulses to effectively permeabilize the cell membranes and enhance the release of intracellular bioactive compounds. The three PEF treatments resulted in distinct phytochemical profiles. Treatment (i) yielded an extract with a TPC of 13.29 ± 0.56 mg GAE/g oil extract, a TFC of 11.20 ± 0.98 mg CE/g oil extract, and a tannin content of 0.11 ± 0.01 mg TT/g oil extract, while its antioxidant capacity, measured by the DPPH assay, was 16.23 ± 0.89 mg TE/g oil extract. Treatment (ii) resulted in a lower TPC of 5.40 ± 0.12 mg GAE/g but a markedly higher TFC of 21.76 ± 1.80 mg CE/g, with a tannin content of 0.02 ± 0.01 mg TT/g and the highest antioxidant capacity at 21.90 ± 1.33 mg TE/g. Treatment (iii) produced an extract with a TPC of 4.43 ± 0.61 mg GAE/g, a TFC of 11.59 ± 0.65 mg CE/g, a tannin content of 0.07 ± 0.01 mg TT/g, and an antioxidant capacity of 16.30 ± 0.40 mg TE/g. As in the pomegranate seed oil model, PEF did not yield measurable oil quantities, but electroporation significantly enhanced the release of soluble phenolic compounds. In fact, PEF (i) treatment exhibited the highest TPC among all tested methods and the third highest antioxidant capacity, highlighting its effectiveness for bioactive compound extraction despite its limited use as a stand-alone oil recovery method. The phenolic recovery was higher than both ultrasound-assisted extractions and hydrodynamic cavitation in this matrix. These findings indicate that PEF holds strong potential as a low-energy technology, particularly when combined with other mechanical methods to enhance extractability and overall bioactive yield in citrus seed valorization.

Table 9 : The values of TPC, TFC, TTC and Antioxidant activity in OS extracted via different green extraction technologies

Extraction technology		TPC (mg GAE/g extract)	TFC (mg CE/g extract)	TTC (mg total tannins/g extract)	DPPH (mg TE/g extract)
UAE bath		7.35 ± 0.91	13.53 ± 1.59	0.14 ± 0.01	11.47 ± 0.44
UAE probe		7.06 ± 1.44	7.64 ± 0.19	0.13 ± 0.02	10.90 ± 0.62
HCAE		7.49 ± 1.13	7.51 ± 0.49	0.69 ± 0.28	19.23 ± 1.21
PEF	(i)	13.29± 0.56	11.20 ± 0.98	0.11 ± 0.01	16.23 ± 0.89
	(ii)	5.40± 0.12	21.76 ± 1.80	0.02 ± 0.01	21.90 ± 1.33
	(iii)	4.43 ± 0.61	11.59 ± 0.65	0.07 ± 0.01	16.30 ± 0.40

4. Conclusions

This study demonstrated that both drying pre-treatment and extraction method significantly affect the phytochemical composition and antioxidant potential of pomegranate and orange seed oil extracts. Among the green extraction technologies evaluated, ultrasound probe-assisted extraction achieved the highest recovery of phenolics and flavonoids within a short processing time. Hydrodynamic cavitation offered moderate efficiency with the advantage of solvent-free operation and scalability. Although PEF did not yield measurable oil extract, effectively enhanced the release of soluble antioxidant compounds, particularly when used as a pre-treatment for aqueous extraction. Freeze-drying emerged as the most suitable drying method for preserving bioactive content, outperforming convective and microwave drying. When applied to orange seeds, the same optimized extraction protocols confirmed their broader utility, although bioactive recoveries were lower compared to pomegranate seeds, likely due to inherent differences in seed matrix composition.

Despite their advantages, green extraction methods showed clear limitations in fatty acid recovery. These limitations arise primarily because such techniques rely on mechanical or non-thermal phenomena (e.g., cavitation, electroporation) and typically use water or mild ethanol–water mixtures as solvents. These solvent systems are not sufficiently lipophilic to

extract neutral and nonpolar lipid fractions effectively. In contrast, conventional methods involving acid hydrolysis and non-polar organic solvents such as petroleum ether and diethyl ether provide a more efficient medium for dissolving and extracting triglycerides and complex fatty acids, including punicic acid. Moreover, the strong solvent–lipid interactions and prolonged contact time in conventional protocols facilitate the breakdown of lipid–protein matrices, enabling more complete lipid recovery.

Therefore, while green extraction technologies offer a sustainable and efficient approach for recovering antioxidant compounds, conventional solvent-based methods remain essential for comprehensive fatty acid profiling and maximum oil recovery. Future work should focus on integrating green techniques with selective solvent strategies or enzymatic pre-treatments to overcome these challenges, advancing the development of eco-friendly and functionally rich seed oil products.

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1.2	29.06.2025	Initial version submitted
1.3	30.06.2025	Final version submitted
2.0	1.07.2025	New draft
2.1	28.07.2025	First version submitted
2.2	30.07.2025	Initial version submitted
2.3	31.07.2025	Final version submitted