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# From lemon peels to bioactive peptides: protein recovery by pressurized liquid extraction and hydrolysates characterization by UHPLC-ESI-QTOF-MS/MS

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ABSTRACT

This study aimed to develop a sustainable and efficient method for protein recovery from lemon peels using Pressurized Liquid Extraction (PLE), optimized through experimental design and response surface methodology. The final protocol, employing 18 % (v/v) ethanol, 110 °C, and a 7-min extraction time, demonstrated high extraction efficiency (66 %) and environmental compatibility, achieving a greenness score of 0.59 (AGREEprep). The optimized protein extract was subsequently hydrolyzed using alcalase and thermolysin, and the resulting peptide fractions were characterized by spectrophotometric methods and by Ultra High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-ESI-QTOF-MS/MS). A total of 58 peptides were identified, 39 of which originated from lemon proteins, along with 19 polyphenols, such as vicenin-2, narirutin or subaphylin, and other compounds (*e.g.*, organic acids, amino acids, and purine nucleosides), revealing a diverse and multifunctional bioactive profile. Bioactivity assays demonstrated that the thermolysin hydrolysate exhibited the highest antioxidant (97 ± 1 % inhibition of hydroxyl radicals), antimicrobial (MIC = 0.75 mg/mL against *Staphylococcus aureus*), and antihypertensive activities (49 ± 3 % angiotensin-converting enzyme inhibition), likely due to the presence of peptides enriched in aromatic amino acids such as phenylalanine and tyrosine.

## 1. Introduction

The global food landscape is undergoing significant transformation, driven by increasing consumer awareness of health and sustainability. There is a growing demand for clean-label and functional food products [1], as well as a marked shift toward plant-based diets, including vegetarian and vegan lifestyles [2,3]. In this context, plant-derived proteins are gaining prominence due to their lower environmental footprint and nutritional benefits [2]. Proteins are essential dietary components that support key metabolic functions, including muscle maintenance, cell signaling, tissue repair, and immune regulation [2]. Beyond their nutritional role, proteins are valued for their functional properties in food systems. Upon hydrolysis, they can release bioactive peptides with diverse biological benefits, such as antioxidant, antimicrobial, anti-inflammatory, and antihypertensive activity [3,4], further enhancing their potential in functional food applications. Consequently, there is growing interest in identifying novel plant-based protein sources to meet increasing nutritional and industrial demands.

Citrus fruits, belonging to the *Rutaceae* family, are among the most widely cultivated and economically significant crops worldwide. Global citrus production has surpassed 100 million tons in 2024, with China, India, and Mexico as leading producers [5]. In particular, Lemons (*Citrus x limon*) and limes (*Citrus x latifolia*) accounted for approximately 23 million tons in 2023/24 [6], highlighting the impact of this sector on the global economy. Still, the consumption of these fruits, whether fresh or processed, generates large volumes of by-products, primarily peels, seeds, pulp, and membranes, which can constitute up to 60 % of the fruit's total weight [7]. Specifically, lemon peel by-products are a rich

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source of bioactive compounds, including polyphenols (*e.g.*, chlorogenic acid, ferulic acid, naringin, naringenin, and hesperidin) [8–11], terpenes (*e.g.*, limonene, β-pinene, γ-terpinene, sabinene, and α-pinene) [12], organic acids (*e.g.*, citric, oxalic, and malic acids) [10,11], and proteins [13]. Hence, the sustainable valorization of lemon peel waste presents a promising approach for converting citrus biomass into high-value bioactive compounds applicable in the agri-food, cosmetic, and nutraceutical industries [11,14]. As such, not only do this strategy helps reduce the environmental and economic impacts of citrus waste, such as resource inefficiency and greenhouse gas emissions, but it also aligns with the principles of the circular economy and biorefinery, promoting the development of plant-based products rich in bioactive and functional compounds [7,15].

Despite their potential, lemon by-products remain an underexplored source of protein. A few studies have reported protein extraction from lemon seeds [16] and pomace [13] using ultrasound- (UAE) and enzyme-assisted (EAE) extraction methods, respectively. However, these approaches require large solvent volumes and sample amount (*e.g.*, 20 g), multiple extraction steps, and extended processing times of up to 16 h. Yet, within the framework of biomass valorization, it is essential to adhere to the principles of Green Extraction; namely, sustainable methodologies that are time- and energy-efficient, minimize the use of organic solvents, and optimize the valorization of natural resources [17]. Among the most promising green extraction techniques is Pressurized Liquid Extraction (PLE), which employs elevated temperatures and pressures to enhance solubility and mass transfer. This results in higher extraction yields and reduced solvent consumption [18]. Additionally, PLE shortens extraction times, preserves thermolabile compounds, is compatible with food-grade solvents, and offers scalability for industrial deployment [4,18]. In this context, some investigations have demonstrated the feasibility of pilot scale PLE-based protocols in recovering bioactive compounds from agri-food residues [19,20]. Notably, advancements have been made in the development of continuous pressurized fluid extraction systems capable of operating under elevated pressure and temperature conditions by utilizing a battery of vessels in series and screw conveyors [21]. These features make it particularly suitable for the recovery of proteins and other bioactive compounds from lemon peels. PLE has already been successfully applied to the extraction of proteins from various horticultural bio-residues, including pomegranate seeds [22] and peels [23], malt rootlets [24], and even lime peels [4], underscoring its robustness, adaptability, and potential for large-scale application.

On top of that, existing studies on protein recovery from lemon by-products have mainly focused on total protein content and physical quality parameters [13,16], rather than on the characterization of the profiles of peptides, proteins and other bioactive compounds present, such as polyphenols. Consequently, data on the composition and bioactive properties of *Citrus limon* peel protein extracts remain scarce, especially those obtained through green chemistry and extraction approaches.

In light of the above, the primary objective of this study was to develop a sustainable and efficient PLE method for recovering proteins from lemon peels, using experimental design and response surface methodology. The optimized protein extract was subsequently subjected to enzymatic hydrolysis using two different proteases, and the resulting peptide fractions were characterized by reversed-phase (RP)/hydrophilic interaction (HILIC) Ultra High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-ESI-QTOF-MS/MS) to identify peptides and other bioactive compounds, including polyphenols and organic acids. Finally, the functionality and potential synergistic effects of the extracts were evaluated through *in vitro* antioxidant, antibacterial, and antihypertensive assays.

## 2. Materials and methods

### 2.1. Solvents, reagents, and standards

All chemicals and solvents used in this study were of analytical grade. Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol and formic acid were sourced from Thermo Fisher Scientific (Waltham, MA, USA). Scharlau (Barcelona, Spain) supplied acetonitrile (ACN), methanol (MeOH), glacial acetic acid, hydrochloric acid (37 %), sodium chloride, and sodium tetraborate. Merck (Darmstadt, Germany) supplied dodecyl sulfate sodium salt (SDS), and Folin-Ciocalteu's phenol reagent (2 N). Additional reagents such as sodium carbonate were obtained from Panreac Química S.L.U. (Barcelona, Spain), and dinitrosalicylic acid (DNS) was purchased from Acros Organics BV (Geel, Belgium).

Sigma-Aldrich (St. Louis, MO, USA) provided 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), gallic acid (98 %), trifluoroacetic acid (TFA), bovine serum albumin (BSA), L-glutathione (GSH), β-mercaptoethanol, Thermolysin (EC 3.4.24.27), *o*-phthalaldehyde (OPA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox (>98 %), D-(+)-glucose (>98 %), ammonium acetate, sodium hydroxide, potassium sodium tartrate, phenol, sodium metabisulfite, angiotensin-converting enzyme (ACE) from rabbit lung (0.25 U), captopril (>98 %), hippuric acid (HA), N-hippuryl-histidyl-leucine (HHL), *p*-iodonitrotetrazolium chloride (INT), and Tryptic-Casein Soy Broth (TSB). Novozymes Spain S.A. (Madrid, Spain) kindly provided alcalase® Pure 2.4 L FG. And the determination of total nitrogen content using the Dumas method was carried out using ethylenediaminetetraacetic acid (EDTA) (Velp Scientifica, Usmate Velate, Italy) as standard.

### 2.2. Citrus sample

*Citrus lemon* L. Burm var. Verna, commonly known as lemon fruits, were procured at their peak ripeness from a local marketplace located in Alcalá de Henares, Madrid. These fruits were carefully cleaned with distilled water to remove any surface contaminants, including dirt, dust, microflora, and pesticide residues. Subsequently, the peels were carefully separated from the fruits using a peeler, ensuring the avoidance of the albedo, and dried in an oven set at 60 °C for 72 h. Following this, the dried peel samples were homogenized into a fine powder using a domestic grinder and preserved in hermetically sealed containers at 4 °C until further use. The moisture content of lemon peels was determined in accordance with the AOAC method 20.013 [25], expressing the results as percentage (mean ± standard deviation ($n$ = 3)). The measured moisture content was (66 ± 1 %).

### 2.3. Pressurized liquid extraction of proteins from lemon peels

#### 2.3.1. Experimental design and optimization

Protein extraction from lemon peels was carried out using an Accelerated Solvent Extraction (ASE) system (ASE 150, Dionex, Sunnyvale, CA, USA) equipped with a 10 mL stainless steel extraction cell. Before extraction, the solvents were degassed in an ultrasonic bath (FB15050, Thermo Fisher Scientific, Waltham, MA, USA) for 45 min. The ASE equipment was preheated for 6 min at 1500 psi and purged with nitrogen for 100 s. A circular cellulose filter (2.5 cm diameter, Whatman) was placed at the bottom of the extraction cell to prevent particulate matter from entering the system.

To optimize protein yield from lemon peels, pressurized liquid extraction (PLE) conditions were refined through experimental design and response surface methodology. A Box-Behnken factorial design was employed, using 2.0000 g of sample, 7.0000 g of sand (LABKEM, Barcelona, Spain) as dispersing agent, with a single extraction cycle. The three independent experimental factors considered were extraction/static time (3–15 min), water-ethanol composition (100:0–20:80, v/v),

and extraction temperature (100–180 °C), resulting in a total of 12 randomized experiments within one block plus three replicates of the central point (9 min, 140 °C, and 40 % EtOH). The response variables used were protein content, determined by Dumas, and Maillard compound formation, measured spectrophotometrically at 420 nm.

Model adequacy was evaluated using the coefficient of determination ($R^2$) and analysis of variance (ANOVA) at 95 % confidence level. Optimal PLE conditions were identified through multiple response analysis (MRA) using a weighted desirability function, aiming to maximize protein extraction while minimizing Maillard product formation, with equal weighting assigned to both responses. The predictive accuracy of the model was validated under optimal conditions (three replicates) by comparing predicted and observed values using a two-tailed *t*-test ($\alpha = 0.05$). In a subsequent phase, the influence of varying the number of extraction cycles (1–3) on the optimal temperature, time, and solvent composition was investigated using the same optimization criteria. All extractions were performed in triplicate. Following the extraction, the resulting 20 mL PLE extracts were stored at -20°C for subsequent Dumas analysis, for no longer than 2 days. For the remaining analyses, the solvent was removed using a vacuum concentrator (Eppendorf AG, Hamburg, Germany), and the resulting dried pellet was preserved at -20°C until further use.

#### 2.3.2. Greenness assessment of the proposed PLE method

The greenness of the procedure was quantitatively assessed using the open-access AGREEprep software [26], a metric tool grounded in the ten principles of green sample preparation. In this study, the AGREEprep evaluation was tailored to reflect the specific characteristics of the PLE method. **Table S1** presents the weighting assigned to each criterion within the AGREEprep software (default weights according [26]), along with the input and justification applied throughout the study. The resulting score, ranging from 0 (least green) to 1 (most green), provided a comprehensive visual and numerical representation of the method's environmental performance.

### 2.4. Determination of the protein content by the Dumas method

Protein content in both the dried raw material and the extracts obtained via PLE was quantified using the Dumas method. Briefly, 100 mg of dried lemon peel or 200–250 mg of liquid PLE extract, along with 150 mg of Super-Absorbent Powder™ (silicon dioxide; Velp Scientifica, Usmate), were accurately weighed using an analytical balance and sealed in tin-pressed capsules. These capsules were then combusted in a Dumas NDA 702 nitrogen analyzer (Velp Scientifica, Usmate). The combustion tube was consistently maintained at 1030°C, while the reduction tube was kept at 650°C. Argon gas was employed as the carrier gas at a flowrate of 190 mL/min, and the total duration of the analysis was set at 400 s. Protein content was estimated by measuring the total nitrogen content. Calibration curves were established through repeated runs using EDTA analytical standard ranging from 0 to 50 mg. Blanks were prepared using the same solvent that was used in the PLE extractions, and all analyses were conducted in triplicate. The total weight of the proteins was calculated applying a nitrogen-to-protein conversion factor of 6.25 [27].

### 2.5. Separation of proteins by SDS-PAGE

Protein separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed following a previously described protocol [28].

### 2.6. Enzymatic hydrolysis of proteins

Protein extracts obtained from lemon peels were hydrolyzed using two different food-grade enzymes: alcalase and thermolysin, following previously reported protocols [4].

Briefly, the protein extract was dissolved in 5 mM borate buffer, adjusted to a pH of 8.0 for thermolysin or 9.0 for alcalase, at a final concentration of 5 mg/mL, using a high-intensity focused ultrasound probe (VCX130, Sonics Vibra-Cell, Hartford, CT, USA) for 5 min at 30 % amplitude. Subsequently, the appropriate enzyme ratio was added: 0.1 g of thermolysin per g of substrate or 0.2 Anson Units per g for alcalase. In parallel, enzyme blanks were prepared using the corresponding borate buffer without protein extract. The mixtures were then incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 70°C and 700 rpm for 1 h (thermolysin) or 3 h (alcalase). Enzymatic hydrolysis was terminated by heating the samples to 100 °C for 10 min while maintaining agitation. The hydrolysates were then centrifuged (Centrifuge 5804, Eppendorf AG, Hamburg, Germany) at 3381×*g* for 10 min at room temperature, and the resulting supernatants were collected for subsequent analyses, including peptide content, degree of hydrolysis, and bioactivity assays. All hydrolysis experiments were performed in quintuplicate.

### 2.7. Characterization of PLE protein extracts and their hydrolysates

#### 2.7.1. Determination of peptide content and hydrolysis degree

Peptide quantification in the hydrolysates was carried out using the *O*-phthalaldehyde (OPA) spectrophotometric assay, following the method described in [29]. Absorbance was measured at 340 nm using a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Hamptoon, New Hampshire, USA). L-glutathione (GSH) was employed as external standard for calibration, with concentrations ranging from 0 to 122 mg/L (n = 5). Results were expressed as milligrams of peptides per milliliter of hydrolyzed extract. All measurements were performed in quintuplicate to ensure reproducibility.

The degree of hydrolysis (HD) was determined by calculating the percentage difference in protein concentration between the initial extract and the resulting hydrolysates, as shown in Equation 1 [28]. Protein quantification was performed according to the Bradford assay [30]. Absorbance was measured at 595 nm using a microplate photometer. For calibration, a standard curve was established using BSA ($n = 6$, 0 to 6 mg/mL). Protein content was expressed as milligrams of protein per milliliter of sample. All measurements were conducted in triplicate to ensure accuracy and reproducibility.

$$\text{HD}\ (\%) = \frac{[proteins]_{initial\ extract}(mg/L) - [proteins]_{hydrolyzed\ extract}\ (mg/L)}{[proteins]_{initial\ extract}\ (mg/L)} \cdot 100 \quad (1)$$

#### 2.7.2. Total phenolic content

The total phenolic content (TPC) of the PLE lemon peel extracts was determined using the Folin-Ciocalteu method [31]. This analysis was applied to several fractions, including the protein extract, the enzymatically treated samples (alcalase and thermolysin hydrolysates), and the filtered protein extract obtained after ultrafiltration. The latter was prepared by passing 500 μL of PLE extract through a 3 kDa centrifugal filter (Amicon® Ultra, Merck, Darmstadt, Germany) at 3381× *g* for 20 min, at room temperature. Gallic acid was used as an external standard, with a calibration range of 0–4 mg/L ($n = 6$). The absorbance of the resulting blue complex was measured at 760 nm using a microplate photometer. Results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE·$g^{-1}$). All measurements were performed in triplicate.

#### 2.7.3. Spectrophotometric estimation of reducing sugars

Reducing sugars were quantified using the dinitrosalicylic acid spectrophotometric method [32]. The tested samples included the initial protein extract; the former filtered through a 3 kDa centrifugal filter, and both hydrolysates. Absorbance was measured at 575 nm using a microplate photometer (UV–Vis Thermo Scientific Multiskan Sky spectrophotometer, Agilent Technologies). The blanks were prepared using

the same solvents as those used in the protein extract and/or the hydrolysis controls, rather than the sample solutions themselves. A calibration curve was established using D-(+)-glucose in the range 0.1–0.5 g/L ($n = 5$). Results were reported as milligrams of glucose equivalents per gram of extract (mg GE/g). All assays were conducted in triplicate.

#### 2.7.4. Peptide identification via UHPLC-ESI-QTOF-MS/MS

Peptides hydrolyzed with alcalase and thermolysin from optimized PLE lemon peel extracts were identified through an untargeted analysis using a high-sensitivity 6530 series Quadrupole-Time-of-Flight (QTOF) mass spectrometer coupled to a 1290 Infinity II UHPLC, both from Agilent Technologies. To cover a broad polarity range, hydrolysates were separated by RP-UHPLC and HILIC. For RP-UHPLC analysis, peptide separation was achieved using a ZORBAX Eclipse Plus C18 analytical column (100 × 2.1 mm, 1.8 μm), paired with a matching guard column (5 × 2.1 mm, 1.8 μm), both supplied by Agilent Technologies (Santa Clara, CA, USA). The mobile phase system consisted of solvent A (0.1 % (v/v) formic acid in water) and solvent B (0.1 % (v/v) formic acid in ACN). The gradient program was optimized as follows: an initial increase from 5 % to 68 % B over 20 min, followed by a rapid ramp to 95 % B within 1 min, held for 1 min, and then returned to 5 % B over 3 min to re-establish baseline conditions. The injection volume was set at 5 μL, with a flow rate of 0.4 mL/min, and the column was maintained at 25 °C [27]. For HILIC, separation was carried out using an Ascentis Express HILIC column (100 × 2.1 mm, 2.7 μm) along with a corresponding guard column (5 × 2.1 mm, 2.7 μm), both from Supelco (Bellefonte, PA, USA). The mobile phases consisted of ACN (solvent A) and 65 mM ammonium acetate in water (solvent B). The mobile phase gradient was programmed to increase from 5 % to 30 % B over 20 min, held at 30 % B for 3 min, and then returned to 5 % B within 2 min. The injection volume was 5 μL, with a flow rate of 0.3 mL/min, and the column temperature was kept constant at 25 °C [27]. Prior to analysis, samples were filtered using regenerated cellulose syringe filters with a pore size of 0.45 μm (Sartorius, Barcelona, Spain). Three independent samples were analyzed, each injected in triplicate.

Peptide profiling was conducted using a high-resolution mass spectrometer operated in positive ionization mode, employing a full scan across the *m/z* range of 100–1700, as outlined elsewhere [4,27]. ESI parameters were 200 V fragmentor voltage, 0 V nozzle voltage, 50 psig nebulizer pressure, 3500 V capillary voltage, 350 °C gas temperature, and 12 L/min drying gas flow. The skimmer voltage was set to 60 V. Internal calibration was performed using the reference standards HP0321 and HP1221, exhibiting *m/z* values of 322.0481 and 1221.9906, respectively. These calibrants were prepared in an ACN-water mixture (95:5, v/v) and introduced directly into the ESI-QTOF system at a flow rate of 15 μL/min. Their continuous infusion enabled real-time monitoring and correction of mass spectrometric fluctuations throughout the analytical run. Data interpretation was carried out using PEAKS Studio version 7 (Bioinformatics Solutions Inc., Waterloo, Canada), which enabled both database-driven identification (via PEAKS DB) and *de novo* sequencing. Peptides identified through this analysis were considered valid if they were consistently detected in at least two out of three independent replicates and exhibited an average local confidence (ALC) score of 85 % or higher, reflecting high sequence reliability. Later, when possible, precursor protein identification was performed against *Citrus limon (Citrus medica* var. *limon)* protein sequences retrieved from the UNIPROT database. All sequences were formatted in FASTA, a standard format for sequence alignment and bioinformatics analysis. The search parameters were defined as follows: fragment ion mass tolerance was set at 0.5 Da, precursor ion tolerance at 10 ppm, and a minimum ALC threshold of 85 % was required. To ensure high confidence in identifications, only peptide matches with a false discovery rate (FDR) below 0.1 % and a -logP score of at least 20 were accepted. Last but not least, sodium adducts were included as potential modifications during the analysis.

#### 2.7.5. Profiling of polyphenols and other metabolites using UHPLC-DAD-ESI-QTOF-MS/MS

Identification of both phenolic compound and other secondary metabolites across both hydrolyzed extracts was conducted using the aforementioned UHPLC-ESI-QTOF-MS/MS coupled online with a diode array detector (DAD). Data acquisition, instrument control, and processing were carried out using Agilent MassHunter Qualitative Analysis Software B.07.00.

Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 analytical column (100 × 2.1 mm, 1.8 μm) paired with a ZORBAX Eclipse Plus C18 guard column (5 × 2.1 mm), both from Agilent. The method followed the protocol described by Domínguez-Rodríguez et al. [10]. The mobile phase system consisted of solvent A (water with 0.1 % (v/v) formic acid) and solvent B (acetonitrile with 0.1 % (v/v) formic acid), applied in a gradient as follows: 0–30 % B from 0 to 7 min, 30–80 % B from 7 to 9 min, 80–100 % B from 9 to 11 min, held at 100 % B from 11 to 13 min, and re-equilibrated to 0 % B during 1 min. The injection volume was 2 μL, with a flow rate of 0.5 mL/min, and the column temperature maintained at 40 °C. Detection wavelengths were set at 200, 280, 350, and 520 nm. Mass spectrometric detection was performed in positive and negative ionization mode, scanning across an *m/z* range of 50–1400. Instrument settings included a capillary voltage of 3000 V, nebulizer pressure of 40 psi, drying gas flow rate of 8 L/min at 300 °C, and a fragmentor voltage of 110 V. The skimmer and octopole voltages were set at 45 V and 750 V, respectively. Sheath gas was delivered at 11 L/min and 350 °C. Three distinct samples were analyzed, with each one subjected to three separate injections. Compound identification was achieved by comparing their MS/MS fragmentation spectra with the literature and with mass spectral information available in the FOODB database (https://foodb.ca/). To assess the reliability of these annotations, confidence levels were assigned according to the five-level Schymanski scale, where level 1 corresponds to confirmation with an authentic standard; level 2a to spectral matches in curated databases; level 2b to matches with literature-reported spectra; level 3 to tentative structural proposals based on fragmentation; level 4 to molecular formula assignment without structural elucidation; and 5 to exact mass detection without further structural characterization [33].

### 2.8. Evaluation of the bioactive potential of protein hydrolysates derived from lemon peels

#### 2.8.1. Antioxidant activity

The antioxidant activity of the hydrolysates was evaluated using three complementary assays: hydroxyl radical inhibition, ABTS radical scavenging, and ferric reducing antioxidant power (FRAP), described by [34]. Positive controls were prepared using Trolox and GSH, while solvent blanks were included for each assay to ensure accuracy. Each sample was analyzed in triplicate, and every replicate was measured at least three times to ensure reproducibility. Results were expressed as percentage inhibition ( %) for hydroxyl radical formation and ABTS radical scavenging activity, and as percentage of reducing power ( %) for the FRAP assay.

#### 2.8.2. Antihypertensive activity

The antihypertensive potential of the hydrolyzed PLE extracts was assessed following the method described by [4], which is based on the enzymatic conversion of the substrate HHL into hippuric acid by ACE. Product and substrate evolution was monitored by HPLC-DAD, using an Agilent system Mod. 1100 Series (Agilent Technologies, Madrid, Spain), equipped with a G1311 pump, a G1379A degasser, a G1316A oven, a G1315B diode array detector, a G1313A autosampler and the Agilent ChemStation software package for data collection and processing. Chromatographic separation was performed on an Ascentis Express Peptide ES-C18 column (2.1 mm x 100 mm, 2.7 μm, Sigma-Aldrich, Saint Louis, MO, USA), maintained at 25°C. The mobile phase consisted of 0.05 % (v/v) TFA in water (solvent A) and 0.05 % (v/v) TFA in

ACN (solvent B), with a gradient elution program as follows: linear increase from 5 % to 60 % B over 10 min, held for 3 min, and returned to initial conditions (5 % B) within 1 min. The injection volume was 10 μL; the flow rate was 0.3 mL/min and the detection was carried out at 228 nm. For sample preparation, 10 μL of ACE 0.05 U/mL was incubated with 17.5 μL of 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl, 2.5 μL of sample or blank, and 5 μL of 1.3 mg/mL HHL in 50 mM HEPES with 300 mM NaCl. The reaction mixture was incubated at 70 °C for 1 h under continuous stirring at 750 rpm. The enzymatic reaction was stopped by adding 20 μL of 1 M HCl. Results were expressed as the percentage of ACE inhibition ( %) based on the reduction in HA peak area relative to the blank.

#### 2.8.3. Antibacterial activity

The antibacterial activity of the hydrolyzed extracts was evaluated against two Gram-negative strains (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027) and one Gram-positive strain (*Staphylococcus aureus* ATCC 29213). The assessment was conducted using the microdilution method in combination with the rapid colorimetric assay based on INT, following the procedure described in [35], with some modifications. Briefly, 80 μL of each hydrolyzed extract was mixed with 100 μL of TSB and 20 μL of bacteria suspension ($1.5 \times 10^6$ CFU (colony forming units)/mL in sterile water), resulting in final sample concentrations ranging from 3 to 0.20 mg/mL. Non-inoculated TSB and aqueous extract were used as negative controls, while the antibiotic streptomycin was used as the positive control. After 24 h of incubation at 37 °C, 40 μL of 0.2 mg/mL INT was added to each well. The development of pink staining indicated the presence of viable microorganisms, facilitating the visual screening of bacterial growth inhibition. Outcomes were expressed as minimum inhibitory concentrations (MIC; mg/mL).

### 2.9. Statistical analysis

The adjustment and multiple response surface analysis of the experimental data, based on the proposed experimental designs and optimization procedures, were conducted using multifactorial analysis of variance (ANOVA). Additionally, differences among the mean values were evaluated using one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test or the Student's *t*-test at a 95 % confidence level, as appropriate. All statistical analyses were performed using Statgraphics 19 software (Statgraphics Technologies, Inc., Rockville, MD, USA).

## 3. Results and discussion

### 3.1. Development of a PLE method for the recovery of proteins from lemon peels

Among the various protein extraction techniques from plant-based matrices, EAE and UAE have been widely used due to their simplicity [4]. Moreover, PLE emerged as a more innovative technique, attracting growing interest due to its high efficiency in protein extraction. This has led to the development of pilot-scale and industrial-scale procedures, further highlighting its potential for large-scale applications [18–21]. As such, it is essential to first establish robust and reproducible operating conditions at the laboratory scale, which will serve as the foundation for subsequent process modelling and scale-up efforts. Since PLE's performance is dependent on several key operational variables, such as solvent composition, extraction temperature, and static time [4,18], this highlights the need for a deeper understanding and systematic optimization of PLE, particularly in the context of the present study. To address this, experimental design and response surface methodology (RSM) were employed. These statistical tools allow for the efficient identification of optimal extraction conditions using a minimum number of experimental runs, while also providing insights into the interactions and effects among the selected factors [36].

For this purpose, a three-level Box-Behnken design incorporating three central replicates was selected. This experimental model was chosen based on prior evidence suggesting a quadratic relationship between the selected parameters and protein extraction yields [4,27]. The inclusion of central replicates enhances the statistical robustness of the design by enabling the evaluation of experimental reproducibility and the identification of curvature or distortion in the response surface, thereby ensuring the reliability and precision of the optimization process [37]. Table 1 presents the set of planned experiments, detailing the experimental factors under investigation along with the selected response variables. These include the protein content (expressed as g of protein per 100 g of dry peel), estimated by the Dumas method, and the formation of Maillard reaction products, assessed by measuring the absorbance of the extract at 420 nm (corresponding to the maximum absorption of melanoidins). The latter response was included due to the high temperatures involved in the extraction process and the abundance of carbohydrates in lemon peels, which can promote Maillard reactions with amino acids, peptides, or proteins present in the sample [4,38]. This reaction may interfere with total nitrogen quantification by the Dumas method and lead to a loss of protein nutritional quality [39], potentially compromising the overall quality of the obtained extracts. Reproducibility of central points (experiments 1, 9, and 14) was considered good (RSD < 3.5 %, for both protein and Maillard products).

Following the experimental design, both responses were fitted to second-order polynomial models as described in Eq. 2:

$$\boldsymbol{Y} = \beta_0 + \beta_1 \boldsymbol{t} + \beta_2 \boldsymbol{T} + \beta_3 \boldsymbol{E} + \beta_{12} \boldsymbol{t\ x\ T} + \beta_{13} \boldsymbol{t\ x\ E} + \beta_{23} \boldsymbol{T\ x\ E} + \beta_{11} \boldsymbol{t}^2 + \beta_{22} \boldsymbol{T}^2 + \beta_{33} \boldsymbol{E}^2 \quad (2)$$

Where $Y$ represents the predicted response (either protein content or absorbance of Maillard compounds), $\beta_0$ is the intercept, and $\beta_1$, $\beta_2$ and $\beta_3$ are the linear coefficients corresponding to static time ($t$), extraction temperature ($T$), and the ethanol proportion in the solvent ($E$), respectively. The terms $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ denote the quadratic coefficients, while $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ represent the interaction coefficients among the experimental factors.

Table 2 presents the parameter values obtained by fitting the experimental data to Eq. 2 for each response. As observed, the model equations exhibited acceptable coefficients of determination ($R^2$), all above 0.8546, and standard errors of estimation (SEE) below 0.15. The protein content estimated via the Dumas method showed comparatively poorer model fits, which may be attributed to the fact that Dumas does

**Table 1**
Box-Behnken experimental design matrix, including normalized factor levels (in parentheses) and experimental responses obtained for protein extraction from lemon peels using PLE.

| Experiment | Factors | | | Responses | |
|---|---|---|---|---|---|
| | *Static time* (min) | *Temperature* (°C) | *EtOH* (%, *v*/*v*) | *Protein content* (*g protein*/*100 g peel*) | *Absorbance at 420 nm* (*AU*) |
| *1* | 9 (0) | 140 (0) | 40 (0) | 5.9 ± 0.2 | 0.177 ± 0.005 |
| *2* | 9 (0) | 180 (1) | 80 (1) | 3.3 ± 0.2 | 0.436 ± 0.001 |
| *3* | 3 (-1) | 140 (0) | 0 (-1) | 4.6 ± 0.3 | 0.077 ± 0.005 |
| *4* | 3 (-1) | 180 (1) | 40 (0) | 7.61 ± 0.01 | 0.45 ± 0.02 |
| *5* | 3 (-1) | 140 (0) | 80 (1) | 1.1 ± 0.3 | 0.173 ± 0.006 |
| *6* | 15 (1) | 140 (0) | 80 (1) | 5.2 ± 0.5 | 0.19 ± 0.02 |
| *7* | 9 (0) | 100 (-1) | 80 (1) | 2.8 ± 0.1 | 0.090 ± 0.005 |
| *8* | 15 (1) | 140 (0) | 0 (-1) | 3.8 ± 0.4 | 0.110 ± 0.005 |
| *9* | 9 (0) | 140 (0) | 40 (0) | 6.1 ± 0.2 | 0.189 ± 0.005 |
| *10* | 15 (1) | 180 (1) | 40 (0) | 6.9 ± 0.5 | 0.55 ± 0.06 |
| *11* | 9 (0) | 180 (1) | 0 (-1) | 6.6 ± 0.9 | 0.35 ± 0.02 |
| *12* | 15 (1) | 100 (-1) | 40 (0) | 4.4 ± 0.4 | 0.092 ± 0.002 |
| *13* | 3 (-1) | 100 (-1) | 40 (0) | 3.5 ± 0.1 | 0.065 ± 0.002 |
| *14* | 9 (0) | 140 (0) | 40 (0) | 6.2 ± 0.4 | 0.187 ± 0.005 |
| *15* | 9 (0) | 100 (-1) | 0 (-1) | 5.7 ± 0.5 | 0.056 ± 0.002 |

**Table 2**
Fitted parameter values taken from the second-order polynomial model described in Eq. (2), goodness-of-fit ($R^2$), standard error of estimate (SEE) and optimal combination of factors from multi-response surface methodology (M-RSM).

| | | | Protein content (g protein/100 g peel) | Absorbance at 420 nm (AU) |
|---|---|---|---|---|
| **Model coefficients** | | | | |
| $\beta_0$ | ***(intercept)*** | | 6.07 | 0.184 |
| $\beta_1$ | ***(static time linear coefficient)*** | | **0.42*** | **0.022*** |
| $\beta_2$ | ***(extraction temperature linear coefficient)*** | | **1.01*** | **0.186*** |
| $\beta_3$ | ***(ethanol proportion linear coefficient)*** | | **-1.06*** | **0.037*** |
| $\beta_{11}$ | ***(static time quadratic coefficient)*** | | **-0.71*** | 0.0042 |
| $\beta_{12}$ | ***(static time-temperature interaction coefficient)*** | | **-0.38*** | **0.0187*** |
| $\beta_{13}$ | ***(static time-ethanol proportion interaction coefficient)*** | | **1.18*** | -0.005 |
| $\beta_{22}$ | ***(extraction temperature quadratic coefficient)*** | | 0.27 | **0.101*** |
| $\beta_{23}$ | ***(temperature-ethanol proportion interaction coefficient)*** | | -0.10 | 0.013 |
| $\beta_{33}$ | ***(ethanol proportion quadratic coefficient)*** | | **-1.73*** | **-0.052*** |
| **Model $R^2$ adjusted** | | | 0.8546 | 0.9816 |
| **SEE** | | | 0.1527 | 0.006 |
| **Best factor combination** | | **Time (min)** | 7 | |
| **M-RSM** | | **Temperature (°C)** | 110 | |
| | | **EtOH (%, v/v)** | 18 | |

* Coefficients with $p$-value $\leq 0.05$ are statistically significant at the 95 % confidence level.

not differentiate between nitrogen sources (including nitrates, nitrites, and organic nitrogen compounds such as those formed through Maillard reactions) [40]. Consequently, the formation of these compounds during the extraction process could lead to an overestimation of protein content in the extracts, thereby reducing the model's predictive accuracy. For this reason, it is essential to monitor the formation of Maillard compounds at 420 nm and to optimize both responses simultaneously.

Regarding the coefficients of the mathematical models within the studied domain, Table 2 revealed that the ethanol proportion ($\beta_3$) and the temperature ($\beta_2$) were the most significant factors influencing both responses, followed by the extraction/static time ($\beta_1$) as the least influential. The temperature and time exhibited a positive effect, whereas the ethanol proportion negatively impacted the protein content while simultaneously promoted the formation of Maillard reaction products, as indicated by a positive coefficient for absorbance response (Table 2). This suggests that minimizing Maillard compound formation is favored in more aqueous media. This observation aligns with the findings of Dai *et al.*, who reported that the rate and extent of the Maillard reaction are higher in organic-water mixtures than in aqueous ones [41]. Notably, the quadratic terms, particularly those for ethanol proportion ($\beta_{33}$) and extraction time ($\beta_{11}$), were significant with respect to protein content, both showing negative coefficients (Table 2). Conversely, the quadratic coefficient for temperature ($\beta_{22}$) had a positive effect on Maillard compound formation, which is consistent with the enhanced reactivity of these compounds at elevated temperatures [39]. The relevance of these quadratic effects is clearly illustrated in the

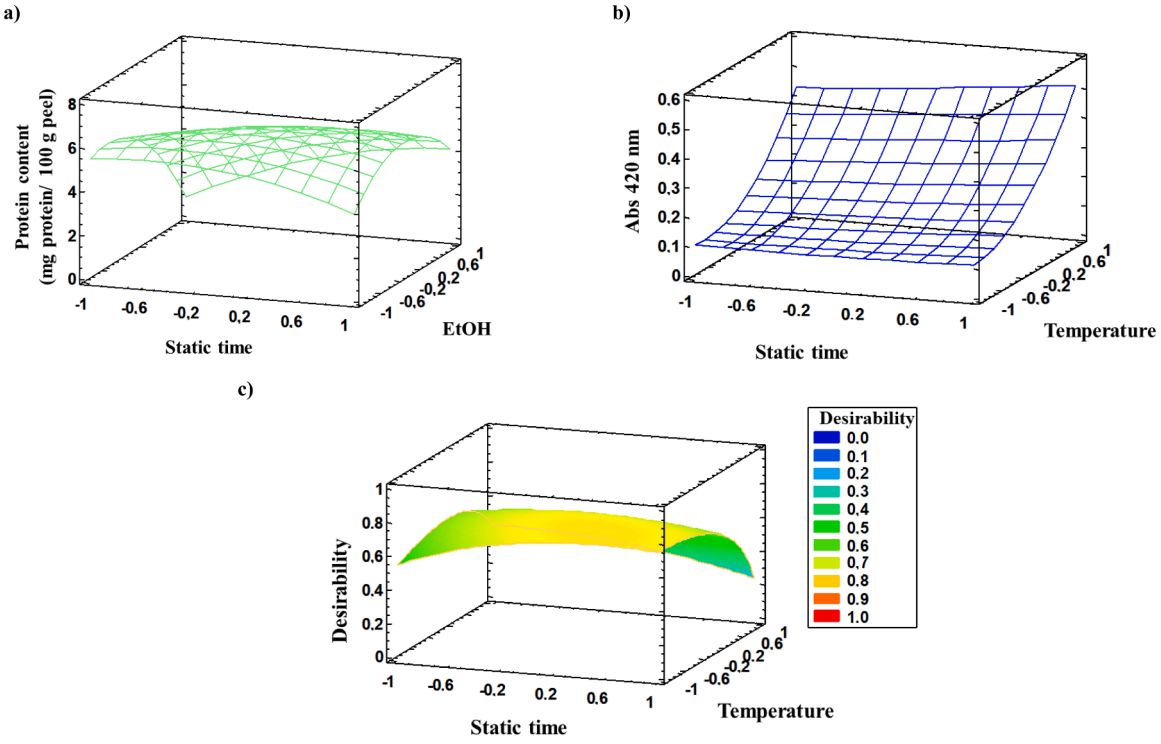


**Fig. 1.** Estimated normalized response surfaces: **a**) protein content (mg protein/100 g peel) at an extraction temperature of 140 °C ($T = 0$); **b**) absorbance at 420 nm, indicative of Maillard compound formation, at an ethanol concentration of 40 % v/v ($E = 0$); and **c**) desirability surface obtained through multiple response optimization, also at 40 % v/v ethanol ($E = 0$).

estimated three-dimensional response surfaces for both protein content and Maillard compound absorbance (Fig. 1), where greater surface distortion highlights the role of interactions, specifically, the interaction between the time and the ethanol proportion ($\beta_{13}$) for the protein content, and between the time and the temperature ($\beta_{12}$) for Maillard compound absorbance at 420 nm.

As previously noted, for carbohydrate-rich citrus matrices, it is essential to apply multiple response surface methodology (M-RSM) to define a single set of optimal PLE conditions. The selected optimization criteria aimed to maximize protein recovery while minimizing absorbance at 420 nm associated with Maillard compounds. The optimal PLE conditions were defined as 7 min of extraction at 110 °C with an EtOH-Water ratio of 18:82 ( %, v/v) (Table 2). Under these conditions, the desirability function reached a value of 0.80, and the predicted values from the model (5.94 mg protein/100 g sample and 0.094 AU) showed no significant differences ($p$-value $>$ 0.05) from the experimental results: 5.7 ± 0.2 mg protein/100 g sample and (9.3 ± 0.6) $x$ $10^{-2}$ AU, with relative deviations below 0.038.

Additionally, the influence of the number of static cycles on protein extraction was assessed, considering that PLE allows for multiple static cycles, which may enhance extraction efficiency [4]. Thus, under the optimal combination of factors, extractions were performed using one, two, and three static cycles. One-way ANOVA followed by LSD test indicated no statistically significant differences ($p$-value $>$ 0.05) in protein content determined by the Dumas method (ranging from 5.8 to 6.12 mg protein/100 g peel). However, a significant increase ($p$-value $<$ 0.05) in Maillard compound formation was observed (ranging from 0.15 to 0.19 AU) with increasing the cycle number. Consequently, a single static cycle was selected as the optimal condition, offering simpler operation, reduced solvent consumption, and shorter processing time.

Under these optimal PLE conditions, a protein extraction yield of 66 ± 3 % was achieved, relative to the total protein content in the starting lemon peel material (8.6 ± 0.2 mg protein/100 $g$ peel). These results highlight the high efficiency of the optimized PLE method, particularly when compared to the UAE procedure adapted from Buket et al., which yielded protein recoveries ranging from 34 % to 53 % in lemon seed matrices [16]. In contrast, Fuso *et al.* reported a protein yield of 93 % from lemon peels using EAE with alcalase at 60 °C for 16 h [13].Yet, such prolonged processing times and the need for enzymatic reagents make the approach less practical and more resource-intensive compared to the rapid, solvent-efficient PLE method developed in this study. Moreover, the performance of the present method is comparable to previously reported PLE protocols for lime peels, which achieved 63–69 % yields under more intensive conditions (elevated temperatures (129–150 °C), and extraction time of up to 11 min) [4,27], further underscoring the current method's practical value and reliability. In addition, the operating variables established at the laboratory scale represent a pivotal foundation for subsequent scale-up investigations and process engineering developments.

### 3.2. Greenness assessment of the optimized PLE method

The analytical procedure developed for protein extraction from lemon peel was evaluated for its environmental sustainability using the Analytical GREEnness metric for sample preparation (AGREEprep). This tool provides a visual representation of the overall greenness performance through a pictogram, where the inner circle displays the final score and the color gradient, ranging from red to green, reflects the level of compliance with green chemistry principles. The evaluation is based on the ten principles of green sample preparation, with individual and overall scores ranging from 0 to 1, where a score of 1 indicates optimal greenness. As can be observed in Fig. 2, the proposed analysis method achieved an optimal score of 0.59 points due to the use of Generally Recognized as Safe (GRAS) materials, the notable use of sustainable, bio-based and renewable materials, and the low waste generation calculated according to the criteria and scores established for this method (see **Table S1**). As such, the method employs only water and ethanol, both environmentally benign solvents, with ethanol classified as GRAS [35], although it remains flammable and irritant. The sample consists of a biodegradable agri-food residue, and sand is used as a natural, reusable support material. Importantly, the extraction solvent is retained within the final extract, contributing to downstream applicability. Nonetheless, there remains room for improvement, as the extraction is carried out in a controlled laboratory environment rather than at the site of waste generation, which may impact the overall logistical sustainability of the process. Additionally, the system operates in semi-automated mode and exhibits an overall high energy demand, driven by both the extraction process, requiring approximately 133 W over a 16-minute PLE cycle (including 7 min for extraction and equipment conditioning); and the subsequent analysis using advanced mass spectrometry techniques.

Collectively, the optimized PLE protocol demonstrated a notable greenness score, indicating its suitability for sustainable protein

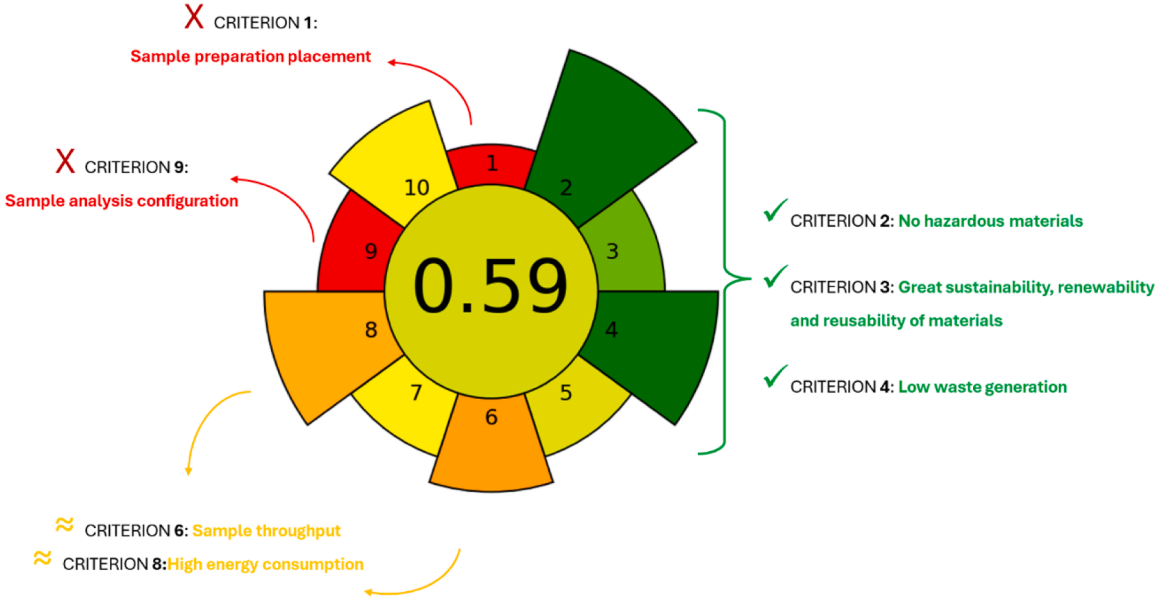


**Fig. 2.** Greenness assessment of the PLE method for protein extraction from lemon peels using the AGREEprep metric.

recovery from lemon by-products. This approach not only valorizes agricultural waste but also aligns with the principles of green chemistry, offering a potentially scalable and eco-friendly alternative for bioactive compound extraction in food and pharmaceutical industries.

### 3.3. Characterization of protein extracts and hydrolysates

#### 3.3.1. Hydrolysis degree and peptide content

Protein extracts obtained under optimal PLE conditions were hydrolyzed to generate bioactive peptides. For this purpose, the hydrolytic activity of two protease enzymes, thermolysin and alcalase, was evaluated under previously established optimal conditions (*Section 2.7.1*). The selection of these enzymes was based on their demonstrated efficiency in releasing peptides from pomegranate and lime peels, as well as their widespread application in the food industry to enhance protein bioactivity and cleave peptide bonds at C-terminal positions, particularly in the case of alcalase [4,27,28].

On the other hand, the hydrolysis degree (HD) was estimated following the procedure described by Hernández-Corroto et al. [28], which relies on protein quantification via the Bradford assay. Due to the limited binding of Coomassie Brilliant Blue to low molecular weight peptides, absorbance decreases as hydrolysis progresses, allowing this method to serve as an indirect indicator of protein hydrolysis [42]. The degrees of hydrolysis and peptide content obtained are summarized in Table 3. As shown, the HD achieved with thermolysin was significantly higher ($p$-value $< 0.05$), exceeding hydrolysis of 95 % of the proteins initially present in the extract. However, in terms of peptide yield, slightly higher values ($p$-value $< 0.05$) were observed in the alcalase hydrolysates, reaching up to 1.9 ± 0.1 mg peptides/mL. Despite this difference, both yields remained within the same order of magnitude (1.6 – 1.9 mg peptides/mL). However, these numerical differences could be explained by several factors. First, the Bradford assay signal depends on the amino acid composition of the proteins present in the initial extract and those remaining after hydrolysis (with a different enzyme), the non-hydrolyzed protein fraction. Indeed, Coomassie Brilliant Blue dye exhibits a stronger affinity for basic amino acids (histidine and arginine) and aromatic amino acids (tryptophan, tyrosine, and phenylalanine) present within the proteins and alcalase and thermolysin cleave proteins at different peptide bonds [42]. Consequently, the proteins remaining after hydrolysis may differ in their amino acid composition and yield different Bradford assay signals, even when the total protein amount is the same. Furthermore, the comparison involves two distinct parameters: the degree of hydrolysis, a relative measure, and the peptide concentration, an absolute measure. These parameters are determined using different analytical methods, namely, the Bradford assay and the OPA method, which rely on separate principles and are subject to different types of analytical interferences and may account for the differences found. These findings are consistent with previous studies, which also reported a lower degree of hydrolysis but higher peptide content in protein hydrolysates from Spanish lime peels treated with AlcalasePURE, compared to those obtained using thermolysin, whose peptides were notably richer in phenylalanine residues [4].

**Table 3**
Degree of hydrolysis, peptide, total polyphenol, and reducing sugar contents in protein extracts and their hydrolysates obtained using thermolysin and alcalase (results expressed as mean ± standard deviation, n ≥ 3).

| | Protein extract | Protein extract filtered through a 3kDa cut-off filter | Alcalase hydrolysate | Thermolysin hydrolysate |
|---|---|---|---|---|
| **Hydrolysis degree (%)** | *N/A* | *N/A* | 86 ± 1 | 97 ± 1* |
| **Peptide content (mg peptides/mL extract)** | *N/A* | *N/A* | 1.9 ± 0.1* | 1.6 ± 0.1 |
| **TPC (mg GAE/g extract)** | 54 ± 2$^{D}$ | 37.8 ± 0.3$^{B}$ | 45.3 ± 0.9$^{C}$ | 20.8 ± 0.9$^{A}$ |
| **Reducing sugars (mg GE/g extract)** | 0.171 ± 0.009$^{B}$ | 0.169 ± 0.005$^{B}$ | 0.14 ± 0.01$^{A}$ | 0.159 ± 0.009$^{B}$ |

N/A: Measurement not applicable or not performed. GAE: Gallic acid equivalent. GE: Glucose equivalent.
* An asterisk indicates statistically significant differences at the 95 % confidence level ($p$-value ≤ 0.05), based on the Student's t-test. Values with different superscript letters indicate significant differences according to one-way ANOVA ($p$-value ≤ 0.05) followed by Fisher's LSD test.

#### 3.3.2. SDS-PAGE of proteins, total phenolic content, and reducing sugars

The protein extract obtained under optimal conditions was characterized by SDS-PAGE (**Fig. S1**). The electrophoretic analysis revealed distinct bands corresponding predominantly to proteins with molecular weights around 25 kDa, along with minor bands at approximately 10–15 kDa and 50 kDa. Consistent with previous studies, citrus peel and seed extracts obtained via UAE exhibited protein bands within the 10–37 kDa range, with no detectable proteins above 50 kDa, particularly in lemon-derived samples [16]. The presence of proteins such as germin-like proteins and superoxide dismutase, with molecular weights around 25 kDa, have been reported in citrus peels, being the most abundant in orange and tangerine [43]. Furthermore, proteomic analysis of lemon peel extract identified the presence of calmodulin-3, thioredoxin, and superoxide dismutase [Cu-Zn] isoform B (12–16 kDa), as well as members of the serine carboxypeptidase S28 family (around 50 kDa) [44].

On the other hand, although the primary objective of this study was the extraction of proteins and the release of bioactive peptides, lemon peels are also rich in other bioactive compounds, such as phenolic compounds and reducing sugars. The presence of extractable polyphenols and reducing sugars in hydroalcoholic media may act synergistically to enhance the bioactivity of the final extracts [11]. Therefore, it was of interest to estimate their free content (*i.e.*, non-protein-bound) using rapid, simple, and cost-effective spectrophotometric methods, specifically, namely, the TPC assay and the DNS method for reducing sugars (Table 3). The total phenolic content and reducing sugar content were determined both in the initial protein extract and in the hydrolysates obtained using proteolytic enzymes. Furthermore, since the TPC assay can be influenced by compounds other than phenolics, including sugars, proteins and peptides [45], the initial protein extract obtained by PLE was filtered through a 3 kDa molecular weight cut-off membrane to separate high-molecular-weight compounds, such as those mentioned, from phenolic compounds, reducing the potential contribution from non-phenolics.

As can be seen in Table 3, TPC value of the unfiltered PLE extract was significantly higher than that of the filtered extract (54 *vs*. 37 mg GAE/g extract), indicating the presence of phenolic compounds in the initial extract and suggesting the contribution of proteins to the TPC signal. Similarly, the hydrolysates showed lower TPC values than the initial extract, with the alcalase hydrolysate exhibiting a significantly higher value compared to the thermolysin hydrolysate (45 *vs*. 20.7 mg GAE/g). This observation further supports the presence of phenolic compounds. However, the lower TPC observed in the hydrolysates compared to the initial extract can be attributed to the oxidative instability of phenolic compounds under the alkaline conditions used during peptide hydrolysis (70 °C, pH 8 for thermolysin and pH 9 for alcalase, with incubation times of 1 and 3 h, respectively). Alkaline pH promotes the deprotonation of phenolic hydroxyl groups, thereby reducing their reactivity with the Folin-Ciocalteu reagent. The decline in TPC is most pronounced during the first hour of incubation, followed by a more gradual decrease over the subsequent 24 h [46]. Additionally, the lower TPC values observed in the thermolysin hydrolysate may also reflect the absence of protein contribution, which is likely more pronounced in the alcalase hydrolysate due to its inferior degree of hydrolysis (Table 3).

Comparable TPC values have been reported by other authors in

extracts obtained from citrus by-products. For instance, TPC values of 54 mg GAE/g have been observed in protein extracts from lime peels obtained via SWE, while hydrolysates produced using alcalase and thermolysin showed TPC values ranging from 46 to 10 mg GAE/g, following a similar trend of TPC reduction after hydrolysis [27]. Similarly, Brezo-Borjan et al. reported TPC values between 45 and 27 mg GAE/g in orange (*Citrus sinensis*) peel extracts obtained by SWE at temperatures up to 200 °C and extraction times ranging from 15 to 60 min [47]. In another study, a PLE method was developed for lemon (*Citrus latifólia* Tan.) pomace, reporting TPC values between 5.09 and 17.65 mg GAE/g [48]. These results confirm not only the effectiveness of the developed PLE method but also highlight the potential of lemon peel residues as a valuable source for the recovery of phenolic compounds, as evidenced by the superior TPC values obtained.

In addition to phenolic compounds, citrus peels also contain structural polysaccharides such as cellulose, hemicellulose, and pectin, as well as reducing sugars like glucose and fructose, which are of industrial interest due to their potential for bioethanol production and their antioxidant capacity, which is linked to their reducing power [32,49]. As shown in Table 3, the reducing sugar content in both the filtered and unfiltered protein extracts, as well as in the thermolysin hydrolysate, did not differ significantly ($p$-value $> 0.05$), with values close to 0.16 mg GE/g extract. In contrast, the alcalase hydrolysate showed a significantly lower reducing sugar content (0.14 mg GE/g extract, $p$-value $< 0.05$), as determined by ANOVA and Fisher's LSD test. This decrease can be attributed to the prolonged exposure of the extract to alkaline conditions (pH 9 for 3 h), which likely intensified the oxidative degradation of reducing sugars, particularly under elevated temperatures and extended reaction times [50]. These results further highlight the potential of thermolysin-derived hydrolysates, which not only retained higher levels of reducing sugars but also exhibited a notably elevated TPC, suggesting their suitability for recovering holistic bioactive compound-rich extracts.

However, spectrophotometric results should be complemented by more selective and robust analytical techniques, as they often suffer from limited selectivity as relying on the overall reactivity of functional groups and are prone to interferences from non-target compounds [45]. In this context, UHPLC-MS/MS provides a powerful and precise tool for the identification of bioactive peptides, polyphenols, and other functional constituents, enabling comprehensive characterization of complex citrus extracts.

### 3.3.4. *Identification of peptides by UHPLC-ESI-QTOF-MS/MS*

In this work, a detailed peptide profiling of *Citrus limon* peel PLE hydrolysates, released using both alcalase and thermolysin, was performed through the integration of two orthogonal chromatographic modes: HILIC and RP-UHPLC. This dual-mode approach enabled a broader and more refined separation of peptides with varying physicochemical characteristics, significantly improving both detection sensitivity and resolution in such complex plant-derived matrices [27]. To date, the scientific literature reports only a single proteomic study focused on lemon peel, which aimed to authenticate the Italian liqueur *Limoncello* using RP-HPLC coupled with LTQ-TOF mass spectrometry [44]. Hence, the present study constitutes a novel and meaningful contribution to the field, not only by expanding the current understanding of lemon peel proteomics through advanced chromatographic strategies, but also by establishing a robust analytical platform for future applications in bioactive peptide discovery and protein-based food characterization.

Fig. 3 showed the total ion chromatogram obtained by RP-UHPLC-MS/MS of the protein hydrolysate generated with alcalase from lemon peel using PLE, and the MS/MS spectrum corresponding to the peptide VVGGAS, identified in this hydrolysate. Furthermore, Table 4 summarized the *de novo* sequences of the peptides identified, along with their characteristics, for hydrolysates produced with alcalase and thermolysin, analyzed using both chromatographic modes (RP and HILIC). Since the *de novo* sequencing tool was unable to distinguish between isoleucine (I) and leucine (L), only isoforms containing L were reported, although both variants were equally plausible.

A total of 58 peptides were identified in the hydrolysates, of which 19 were also detected in the hydrolysis blanks, suggesting that they may originate from the proteolytic enzymes used in the process [4,27]. Specifically, 9 peptides matched sequences from alcalase and 10 from thermolysin. These peptides have been marked with an asterisk in

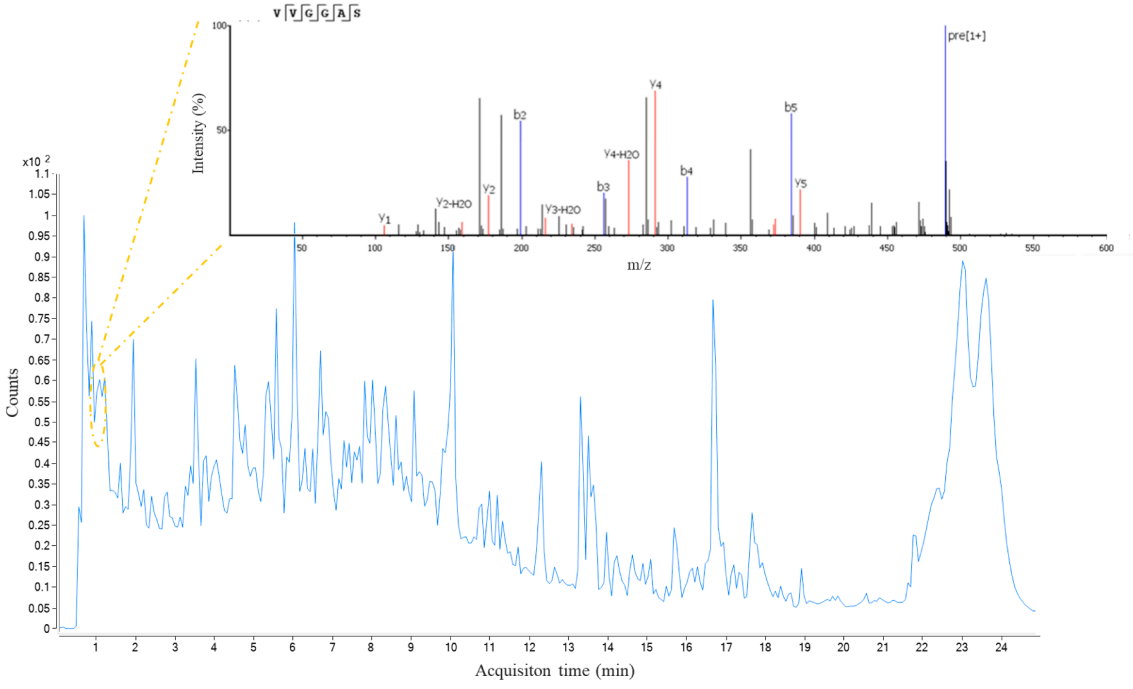


**Fig. 3.** Total ion chromatogram of the hydrolysate obtained by alcalase-mediated enzymatic digestion of a lemon peel protein extract produced via optimal PLE conditions, and mass spectrum of the peptide VVGGAS, identified by RP-UHPLC-QTOF-MS/MS.

**Table 4**
Peptide sequences, retention time, molecular weight, average local confidence scores (ALC), mass accuracy (ppm), and corresponding precursor proteins identified via *de novo* sequencing and UniProt database annotation from PLE lemon peel hydrolysates analyzed using RP-UHPLC-ESI-QTOF-MS/MS and HILIC-ESI-QTOF-MS/MS.

| Number | LC mode | Hydrolysate | Peptide sequence | Retention time (min) | Molecular mass (Da) | ALC (%) | Mass accuracy (ppm) | Parent protein (accession number) |
|---|---|---|---|---|---|---|---|---|
| 1 | **RP** | **Alcalase** | VAGAA | 1.22 | 387.2118 | 86 | -7.6 | |
| 2 | | | VVGGAS | 1.39 | 488.2595 | 89 | -4.1 | RABGTPase (A0A1S8AD82) |
| 3 | | | LSAS* | 1.45 | 376.1958 | 96 | -5.2 | |
| 4 | | | YYGK* | 1.45 | 529.2537 | 94 | -2.1 | |
| 5 | | | VVGQ | 1.75 | 401.2274 | 88 | -1.6 | |
| 6 | | | LLAK | 1.85 | 443.3107 | 99 | -5.1 | |
| 7 | | | VVGGA | 1.97 | 401.2274 | 89 | -5.8 | |
| 8 | | | ISNAS | 2.19 | 490.2387 | 90 | -2.0 | Importin alpha isoform (A0A1S8ABS5) |
| 9 | | | SLGGAS* | 2.25 | 490.2387 | 97 | -3.5 | |
| 10 | | | SHPDLN | 2.30 | 681.3082 | 91 | -1.6 | |
| 11 | | | VVGL | 3.70 | 386.2529 | 86 | -4.6 | |
| 12 | | | LNVE* | 3.83 | 473.2485 | 96 | -6.0 | |
| 13 | | | APGAGVY | 4.63 | 633.3122 | 95 | -4.5 | |
| 14 | | | VAAL* | 4.68 | 372.2372 | 94 | -8.5 | |
| 15 | | | VFPQ | 4.79 | 489.2587 | 96 | -4.8 | |
| 16 | | | MTESA | 4.82 | 537.2104 | 90 | -5,6 | Uncharacterized protein |
| 17 | | | GLLN | 4.88 | 415.2431 | 87 | -4.1 | |
| 18 | | | TLGYPA | 5.32 | 620.3170 | 90 | -3.5 | |
| 19 | | | SVGAEL* | 5.39 | 574.2962 | 96 | -2.7 | |
| 20 | | | SVYGPV | 5.42 | 620.3170 | 88 | -3.5 | |
| 21 | | | VAVL* | 5.91 | 400.2686 | 93 | -5.9 | |
| 22 | | | VGVVV | 5.98 | 471.3057 | 86 | -7.1 | |
| 23 | | | GLPLLK* | 6.08 | 639.4130 | 95 | -4.9 | |
| 24 | | | AVEWATTN | 6.40 | 890.4134 | 94 | -1.5 | |
| 25 | | | LGEWATTN | 6.41 | 890.4134 | 85 | -1.5 | |
| 26 | | | YVGF | 6.78 | 484.2322 | 92 | -3.3 | |
| 27 | | | IGLHF | 7.2 | 585.3275 | | -5,3 | Photosystem II protein D1 (A0A1W6C9E2) |
| 28 | | | ALLL* | 7.86 | 428.2999 | 98 | -5.3 | |
| 29 | | | LALLL | 8.99 | 541.3839 | 90 | -4.1 | |
| 30 | | | LAGLPP | 10.27 | 566.3428 | 85 | -3.7 | |
| 31 | | | VGVAEM | 11.01 | 604.2891 | 89 | -1.0 | Importin alpha isoform (A0A1S8ABS5) |
| 32 | | **Thermolysin** | LANT | 1.23 | 417.2224 | 88 | 2.5 | |
| 33 | | | LNTT | 1.36 | 447.2329 | 90 | -4.9 | |
| 34 | | | MSDPAK* | 1.46 | 647.2949 | 98 | -0.3 | |
| 35 | | | LGED | 1.62 | 432.1856 | 94 | -1.7 | |
| 36 | | | VLGDQKN | 1.64 | 772.4079 | 90 | -4.9 | |
| 37 | | | LTGTST* | 1.70 | 578.2991 | 95 | -3.5 | |
| 38 | | | LTPTSN | 2.18 | 631.3177 | 98 | -4.0 | |
| 39 | | | LTPT | 2.45 | 430.2427 | 85 | -2.9 | |
| 40 | | | TLPGS* | 3.31 | 473.2485 | 93 | -0.7 | |
| 41 | | | LTGTSTVG | 3.51 | 734.3810 | 88 | -1.8 | |
| 42 | | | VYTGP | 3.77 | 535.2642 | 85 | -1.3 | |
| 43 | | | LTQY* | 3.84 | 523.2642 | 93 | -2.7 | |
| 44 | | | LDVVAEH | 4.30 | 781.3970 | 91 | -1.6 | |
| 45 | | | LNTTY* | 4.36 | 610.2963 | 91 | -3.3 | |
| 46 | | | FDAVGVK | 4.63 | 734.3962 | 97 | -3.9 | |
| 47 | | | VTYDY* | 5.22 | 659.2802 | 97 | -2.5 | |
| 48 | | | LFGT | 5.55 | 436.2322 | 86 | -2.4 | |
| 49 | | | LSGGLDVVAHE* | 5.75 | 1095.5559 | 96 | -3.5 | |
| 50 | | | LVLEGT | 5.80 | 630.3588 | 90 | -2.2 | |
| 51 | | | LNPDF | 6.04 | 604.2856 | 91 | 4.4 | |
| 52 | | | LVEF | 7.14 | 506.2740 | 97 | -2.7 | |
| 53 | | | LSDLFGT* | 7.54 | 751.3752 | 92 | -2.9 | |
| 54 | | | LVEFY* | 7.80 | 669.3373 | 93 | -5.8 | |
| 55 | **HILIC** | **Alcalase** | LALGPP | 3.02 | 566.3428 | 89 | -5.3 | |
| 28 | | | ALLL* | 9.55 | 428.2999 | 98 | -1.5 | |
| 21 | | | VAVL* | 10.30 | 400.2686 | 95 | 1.0 | |
| 56 | | | GYSPN | 11.43 | 536.2231 | 91 | 2.3 | R2R3-MYB family transcription factor (A0A411PZP7) |
| 15 | | | VFPQ | 11.46 | 489.2587 | 97 | 5.0 | |
| 13 | | | APGAGVY | 17.10 | 633.3122 | 96 | 0.3 | |
| 55 | | **Thermolysin** | LALGPP | 1.31 | 566.3428 | 90 | -6.9 | |
| 16 | | | MTESA | 4.82 | 537.2104 | 90 | -5.6 | Uncharacterized protein |
| 48 | | | LFGT | 9.12 | 436.2322 | 97 | 1.8 | |
| 43 | | | LTQY* | 11.14 | 566.3428 | 90 | -6.9 | |
| 57 | | | LVLPSG | 11.68 | 584.3533 | 95 | -2.2 | |
| 42 | | | VYTGP | 12.21 | 535.2642 | 91 | 0.5 | |
| 58 | | | LLNAK* | 20.74 | 557.3537 | 85 | -2.8 | NB-ARC domain-containing disease resistent protein (A0A221J431) |

* Peptides matching sequence fragments of the thermolysin or alcalase sequence used in the hydrolytic process of the PLE protein extract.

Table 4, including examples such as VAVL (n° 21) and LTQY (n° 43), among others. While the identification and characterization of peptides derived specifically from lemon peels remains a central focus, it is relevant to adopt a comprehensive approach that considers all peptide constituents present in the extract. Indeed, separating hydrolysates components (in this case lemon peptides and those resulting from enzyme autodigestion) is neither economically feasible nor aligned with sustainable processing strategies. Further to this, enzyme-derived peptides could contribute to the overall bioactivity and functional potential of the final hydrolysate [21,27].

Additionally, a higher number of peptides (93 %) were detected using RP-LC-MS/MS, in contrast to HILIC-MS/MS, which may reflect the physicochemical properties of the peptides. RP-LC favors the retention and separation of apolar or hydrophobic peptides, as evidenced by the frequent presence of residues such as L/I and V. Moreover, RP-LC mobile phases are generally more compatible with ESI, resulting in improved ionization efficiency and enhanced peptide annotation rates. In contrast, HILIC-MS/MS analysis identified 13 peptides, four of which were exclusive to this mode: LALGPP (n° 55) and GYSPN (n° 56) in the alcalase hydrolysate, and LVLPSG (n° 57) and LLNAK (n° 58) in the thermolysin hydrolysate. Such peptides contain polar amino acids such as tyrosine (Y), serine (S), and lysine (K) (Table 4). Notably, several peptides were identified in both RP and HILIC: APGAGVY (n° 13) and VFPQ (n° 15) in alcalase hydrolysates, and LFGT (n° 48) and VYTGP (n° 42) in those from thermolysin. Interestingly, the peptide MTESA (n° 16) was detected by HILIC in the alcalase hydrolysate and by RP in the thermolysin hydrolysate.

Particularly, 33 peptides were identified in the alcalase hydrolysate and 25 in the thermolysin hydrolysate, with no overlap between them, reflecting distinct cleavage preferences. Thermolysin-generated peptides predominantly contained hydrophobic residues such as L, phenylalanine (F), valine (V), I, alanine (A), and methionine (M), showing a higher selectivity for hydrophobic cleavage sites [4]. In contrast, alcalase produced a more diverse set of peptides, indicating broader cleavage activity and thus lower selectivity. Moreover, peptides generated by alcalase were characterized by a higher content of nonpolar aliphatic amino acids such as A, proline (P), I, and the slightly polar aromatic tryptophan (W). In contrast, peptides from thermolysin were enriched in aromatic amino acids such as F and Y (Table 4), both of which are associated with antioxidant properties [27], reinforcing the bioactive potential of the peptides obtained.

Further to this, a comparative analysis was conducted using the Food-Derived Bioactive Peptides (FDBP) database (http://www.cqudfbp.net). This research revealed that peptide FDAVGVK (n° 46) from the thermolysin hydrolysate has been fully identified and reported to possess antihypertensive activity (database identifier DFBPACEI1728). Additionally, LANT (n° 32) and LTPT (n° 39) were partially matched as fragments of longer sequences with documented antimicrobial and ACE inhibitory activities, corresponding to identifiers DFBPAMIC0094 and DFBPACEI0895, respectively. In the alcalase hydrolysate, APGAGVY (n° 13) was as a fully characterized sequence with confirmed antihypertensive and antioxidant activity under the identifiers DFBPACEI1371 and DFBPANOX0396, respectively. Additionally, six peptides (*LSAS* (n° *3), VVGQ* (n° *5), VVGL* (n° *11), VAAL* (n° *14), GLLN* (n° *17), and VAVL* (n° *21)*) were partially identified through sequence comparison with entries in FDBP database. These were found to be fragments of longer peptide sequences previously associated with antimicrobial (DFBPAMIC0356 for VVGQ, DFBPAMIC0380 for GLLN, and DFBPAMIC0358 for VAVL), antioxidant (DFBPANOX0155 and DFBPANOX0156 for VAVL), anticancer (DFBPANCA0049 for VVGQ, DFBPANCA0666 for VVGL, DFBPANCA0841 for VAAL), and ACE (DFBPACEI1733 for LSAS) inhibitory effects. Of these, three were derived from alcalase digestion blanks (LSAS, VAAL and VAVL). Although partial identification does not confirm that these peptides exhibit the same bioactivities as those reported in the FDBP database, their presence suggests a promising bioactive potential. However, further in-depth studies are warranted to validate these preliminary findings and elucidate the correlation between structure and biological function of the identified peptide sequences [27].

Regarding the size of the peptides identified, it is well known that small peptides tend to exhibit greater bioactivity, particularly when they are rich in hydrophobic and aromatic amino acids [34,44]. In this study, over 61 % of the peptides identified were composed of 4–5 amino acids, 23 % had 6 residues, 13 % had 7–8, and only one peptide, LSGGLDVVAHE (n° 49, derived from thermolysin), contained 11 amino acids. This indicated that nearly 99 % of the peptides had a molecular weight below 1 kDa, which may enhance their bioaccessibility and bioactivity.

Compared to bibliographic data, several of these peptides, such as SHPDLN (n° 10), APGAGVY (n° 13), VFPQ (n° 15), LNTT (n° 33), LGED (n° 35), VLGDQKN (n° 36), LTPTSN (n° 38), VYTGP (n° 42), FDAVGVK (n° 46), LFGT (n° 48), LNPDF (n° 51), LVEF (n° 52), LALGPP (n° 55), LVLPSG (n° 57), had been previously reported in lime peels hydrolyzed using alcalase and thermolysin [4,27], suggesting that they may be characteristic of both the hydrolases involved and the *Citrus* genus. By contrast, no overlapping peptides were detected in comparison with the proteomic study of trypsin-digested lemon peel peptides, which showed cleavage at the C-terminal of R and K residues, resulting in longer peptides (>1 kDa) [44]. Hence, this supports the idea that peptides obtained with alcalase and thermolysin are more suited for bioactivity-oriented applications.

Ultimately, in some cases, peptides were successfully assigned to their precursor proteins using the *Citrus* x *limon* sequence. For instance, VVGGAS (n° 2) was part of the RAB GTPase protein, ISNAS (n° 8) and VGVAEM (n° 31) were derived from importin alpha isoform, both involved in transport functions, and IGLHF (n° 27) was associated with the photosystem II protein D1, which plays a role in oxidoreductase activity [51].

### 3.3.5. Identification of polyphenols and other low molecular weight compounds by UHPLC-ESI-QTOF-MS/MS

Given that the optimized PLE methodology is non-selective for proteins and may co-extract other bioactive compounds with potential synergistic effects contributing to the added value and bioactivity of the lemon peel protein hydrolysates, a broader identification of bioactives was conducted using the approach described in *Section 2.7.5*.

**Fig. S2** displays the RP-UHPLC-DAD-QTOF-MS/MS chromatogram at 280 nm of the alcalase-generated protein hydrolysate from lemon peel, along with the MS/MS spectrum of the most representative polyphenol. Additionally, Table 5 presents the profile of additional bioactive compounds tentatively identified through non-targeted UHPLC-MS/MS analysis in both positive and negative ionization modes. A total of 27 compounds were tentatively identified in the protein hydrolysates of lemon peel, based on available literature for *C. limon* [11,52–54], *C. reticulata* [55], *C. aurantium* [56], *C. aurantifolia* [27], and MS/MS spectral comparison with the FOODB mass spectrometry database. According to the Schymanski classification, 18 compounds were assigned level 3 (tentative structure based on fragmentation information from literature), while 9 compounds matched MS/MS spectra in FOODB and were assigned level 2a (Table 5).

A total of 18 compounds were tentatively identified in negative ionization mode, while 9 were detected in positive mode. All bioactive compounds were found in both hydrolysates, except for vanillic acid hexoside, which was exclusive to the alcalase hydrolysate, and narirutin, only detected in the thermolysin hydrolysate (Table 5).

Regarding the nature of the tentatively identified compounds, three organic acids commonly reported in citrus, such as citric and malic acids, were detected, both known for their antimicrobial and antioxidant activities [11,52,55]. Additionally, three amino acids were tentatively identified: two proteinogenic (tryptophan and phenylalanine) and one non-proteinogenic, pipecolic acid, a plant-derived metabolite involved in resistance to biotic and abiotic stress [27,56]. Minor components

**Table 5**
Retention time and MS data for the tentative identification of polyphenols and other constituents in thermolysin and alcalase hydrolysates via RP-UHPLC-ESI-QTOF-MS/MS.

| Number | Retention time (min) | Precursor *m/z* | Adduct | MS/MS main fragments (*m/z*) | Ionization voltage (V) | Molecular formula | Monoisotopic mass | Proposed compound | Compound class | ID level (Schymanski) | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.6 | 191.0578 | $[M-H]^-$ | **85**; 93; 59 | 20 | $C_7H_{12}O_6$ | 192.0633 | Quinic acid | Organic acid | 3 | [52] |
| 2 | 0.7 | 133.0169 | $[M-H]^-$ | **71**; 73; 115 | 20 | $C_4H_6O_5$ | 134.0215 | Malic acid | Organic acid | 3 | [52] |
| 3 | 0.9 | 191.0326 | $[M-H]^-$ | **111;** 85; 87 | 20 | $C_6H_8O_7$ | 192.0270 | Citric acid | Organic acid | 3 | [52] |
| 4 | 1.2 | 130.0513 | $[M+H]^+$ | **84**; 56 | 20 | $C_6H_{11}NO_2$ | 129.0789 | Pipecolic acid | Amino acid | 3 | [27, 56] |
| 5 | 2.1 | 268.1057 | $[M+H]^+$ | **136**; 119 | 40 | $C_{10}H_{13}N_5O_4$ | 267.0967 | Adenosine | Purine nucleoside | 3 | [27, 56] |
| 6 | 2.4 | 284.1148 | $[M+H]^+$ | **152**; 135 | 40 | $C_{10}H_{13}N_5O_5$ | 283.0961 | Hydroxyadenosine | Purine nucleoside | 3 | [27, 56] |
| 7 | 2.7 | 166.0847 | $[M+H]^+$ | **77**; 103; 120 | 40 | $C_9H_{11}NO_2$ | 165.0789 | Phenylalanine | Amino acid | 3 | [56] |
| 8 | 3.3 | 329.0932 | $[M-H]^-$ | **152**; 167; 108 | **20** | $C_{14}H_{18}O_9$ | 330.0139 | Vanillic acid hexoside* | Polyphenol (Phenolic acid) | 3 | [55] |
| 9 | 4.2 | 265.1532 | $[M+H]^+$ | 117; 149; **89;** 72 | 40 | $C_{10}H_{20}N_2O_3$ | 264.1473 | Subaphylin | Polyphenol (Phenolic acid) | 2a | [27] FooDB |
| 10 | 4.7 | 205.0950 | $[M+H]^+$ | **146**; 118; 188 | 20 | $C_{11}H_{12}N_2O_2$ | 204.0899 | Tryptophan | Amino acid | 2a | [27] FooDB |
| 11 | 5.1 | 593.1560 | $[M-H]^-$ | **353**; 383 | 40 | $C_{27}H_{30}O_{15}$ | 594.1584 | Vicenin-2 (apigenin 6,8-di-C-glucoside) | Polyphenol (Flavonoid) | 2a | [54] FooDB |
| 12 | 5.4 | 623.1701 | $[M-H]^-$ | **383**; 312 | 40 | $C_{28}H_{32}O_{16}$ | 624.1690 | Diosmetin 6,8-di-C-glucoside (leucenin 2,4-metileter) | Polyphenol (Flavonoid) | 3 | [27, 53] |
| 13 | 5.6 | 163.0426 | $[M-H]^-$ | **119**; 93 | 20 | $C_9H_8O_3$ | 164.0743 | Coumaric acid | Polyphenol (Phenolic acid) | 2a | [54] FooDB |
| 14 | 5.7 | 357.1216 | $[M-H]^-$ | **151**; 136; 195 | 40 | $C_{16}H_{22}O_9$ | 358.1263 | Dihydroferulic acid glucoside | Polyphenol (Phenolic acid) | 3 | [27, 52] |
| 15 | 6.0 | 595.1671 | $[M-H]^-$ | **151**; 135; 287 | 40 | $C_{27}H_{32}O_{15}$ | 596.1741 | Eriocitrin | Polyphenol (Flavonoid) | 2a | [53] FooDB |
| 16 | 6.0 | 563.1459 | $[M-H]^-$ | **293**; 59 | 40 | $C_6H_{28}O_{14}$ | 564.1479 | Vitexin-O-xyloside | Polyphenol (Flavonoid) | 3 | [27, 52] |
| 17 | 6.2 | 593.1560 | $[M-H]^-$ | **285**; 151 | 40 | $C_{27}H_{30}O_{15}$ | 594.1583 | Luteolin-neohesperidoside | Polyphenol (Flavonoid) | 3 | [52] |
| 18 | 6.2 | 595.1696 | $[M+H]^+$ | **287**; 313; 153 | 40 | $C_{28}H_{34}O_{14}$ | 594.1949 | Poncirin | Polyphenol (Flavonoid) | 2a | [52] FooDB |
| 19 | 6.7 | 653.178 | $[M-H]^-$ | **329**; 330; 345 | 40 | $C_{29}H_{34}O_{17}$ | 654.179 | Limocitrin-neohesperidoside | Polyphenol (Flavonoid) | 3 | [52] |
| 20 | 6.7 | 581.1933 | $[M-H]^-$ | **273**; 195; 153 | 40 | $C_{27}H_{32}O_{14}$ | 582.1875 | Naringenin-7-O-rutinoside (narirutin)** | Polyphenol (Flavonoid) | 2a | [52] FooDB |
| 21 | 6.8 | 195.0691 | $[M-H]^-$ | **121**; 136; 151 | 20 | $C_{10}H_{12}O_4$ | 196.0736 | Dihydroferulic acid | Polyphenol (Phenolic acid) | 2a | [54] FooDB |
| 22 | 6.9 | 607.1714 | $[M-H]^-$ | **299**; 284 | 40 | $C_{28}H_{32}O_{15}$ | 608.1741 | Diosmin (diosmetin 7-rutinoside) | Polyphenol (Flavonoid) | 2a | [53] FooDB |
| 23 | 7.0 | 609.1785 | $[M+H]^+$ | **301**; 153; 85 | 40 | $C_{28}H_{32}O_{16}$ | 608.1741 | Diosmetin-O-glucoside-O-rhamnoside | Polyphenol (Flavonoid) | 3 | [27, 56] |
| 24 | 7.1 | 609.187 | $[M-H]^-$ | 301 | 40 | $C_{28}H_{34}O_{15}$ | 610.1898 | Hesperidin/ Neohesperdin | Polyphenol (Flavonoid) | 3 | [53] |
| 25 | 7.2 | 651.1607 | $[M-H]^-$ | **345.** 549; 507 | 20 | $C_{29}H_{32}O_{17}$ | 652.1639 | Limocitrin-HMG-glucoside | Polyphenol (Flavonoid) | 3 | [11, 52] |
| 26 | 7.3 | 681.1725 | $[M-H]^-$ | **360.** 375; 576 | 40 | $C_{30}H_{34}O_{18}$ | 682.1745 | Limocitrol-glucoside-HMG | Polyphenol (Flavonoid) | 3 | [11, 52] |
| 27 | 7.6 | 697.1936 | $[M+H]^+$ | **391**; 376; 361 | 40 | $C_{31}H_{36}O_{18}$ | 696.1902 | Haploside C | Polyphenol (Flavonoid) | 3 | [27] |

HMG = 3-hydroxy-3-methylglutaryl glucoside. The most abundant MS/MS fragments are highlighted in bold. Experimental precursor *m/z* values are reported as measured by high-resolution MS, while theoretical monoisotopic masses were calculated based on the proposed molecular formulas.

* Indicates compounds identified exclusively in the alcalase hydrolysate, while

** indicates compounds identified only in the thermolysin hydrolysate.

included purine nucleosides such as adenosine and hydroxyadenosine, which play essential roles in biochemical processes and have potential therapeutic applications, including neuromodulation in the central nervous system [27].

Polyphenols represented the predominant group of non-peptidic bioactives, accounting for approximately 70 % of the total identified compounds. These phytochemicals are known for their broad spectrum of biological activities, adding significant value to the protein hydrolysate. Among them, five phenolic acids were tentatively identified, including well-known coumaric acid and dihydroferulic acid [11,45,55], as well as less-studied compounds such as subaphyllin [27]. Citrus peels are a recognized source of phenolic acids, although in lower abundance compared to flavonoids. Within this group, hydroxycinnamic acids (*e.g.*, ferulic and coumaric acids) are more prevalent than hydroxybenzoic acids [11,54], and have shown α-glucosidase and nitric oxide inhibitory activities, supporting their potential therapeutic applications [11].

Flavonoids constituted the most abundant subclass of polyphenols. Among them, flavones were predominant, with derivatives of apigenin, diosmetin, vitexin, and limocitrin being tentatively identified (Table 5). Notably, hesperidin/neohesperidin was determined as one of the major phenolic compounds in citrus peels (**Fig. S2**). Due to its high abundance, hesperidin has been extensively studied and is associated with various health-promoting properties, including antioxidant, anticancer, anti-inflammatory, cardioprotective, and antiviral effects [27,45,54,55].

### 3.4. Evaluation of the bioactive potential of protein hydrolysates

To evaluate the potential of lemon peel hydrolysates as functional ingredients for applications in the food and nutraceutical industries, alcalase and thermolysin hydrolysates from the protein extract obtained under optimal PLE conditions, were characterized and compared in terms of their antioxidant, antimicrobial, and antihypertensive activities. These *in vitro* assays were not performed on the protein extract, as intact proteins have shown limited bioactive potential, attributed to their susceptibility to gastrointestinal degradation and poor absorption through the intestinal lumen due to their molecular size [22,57]. Therefore, the study focused on protein hydrolysates, which are generally more relevant for assessing bioactivity.

The antioxidant capacity of the hydrolysates was evaluated using three complementary assays: ABTS radical scavenging, hydroxyl radical inhibition, and FRAP. The results are presented in Fig. 4**a**. Thermolysin hydrolysate exhibited significantly higher antioxidant activity across all assays ($p$-value $< 0.05$), particularly in hydroxyl radical inhibition, reaching up to 97 ± 1 %. This was followed by its ability to reduce Fe (III) and inhibit radical formation, with FRAP inhibition values around 70 ± 1 %. These assays are especially relevant, as iron catalyzes free radical generation via the Fenton reaction, and hydroxyl radicals are among the most reactive ROS in biological systems [11], highlighting the potential of both hydrolysates, particularly the thermolysin-derived one, for nutraceutical applications. The superior antioxidant performance of the thermolysin hydrolysate may be attributed to the presence of peptides enriched in aromatic amino acids such as F and Y [27,34], the latter bearing a hydroxyl group that enhances radical scavenging capacity, resembling polyphenolic structures. Yet, the antioxidant peptide APGAGVY, previously identified in *Olea europaea* seed extracts [27], was also detected in the alcalase hydrolysate (Table 4), potentially contributing to its activity. In addition to peptides, other bioactives such as citric and malic acids, known for their metal-chelating properties [11], may also enhance the FRAP. Furthermore, polyphenols including hesperidin, coumaric acid, diosmetin, apigenin, and their derivatives, all recognized for their strong antioxidant potential in citrus peels [11,45,54], likely contribute synergistically to the observed effects.

On the other hand, the antimicrobial potential of the hydrolysates was evaluated (Fig. 4**b**). No inhibition was observed for the Gram-negative *P. aeuroginosa* strain at the highest concentration tested (3

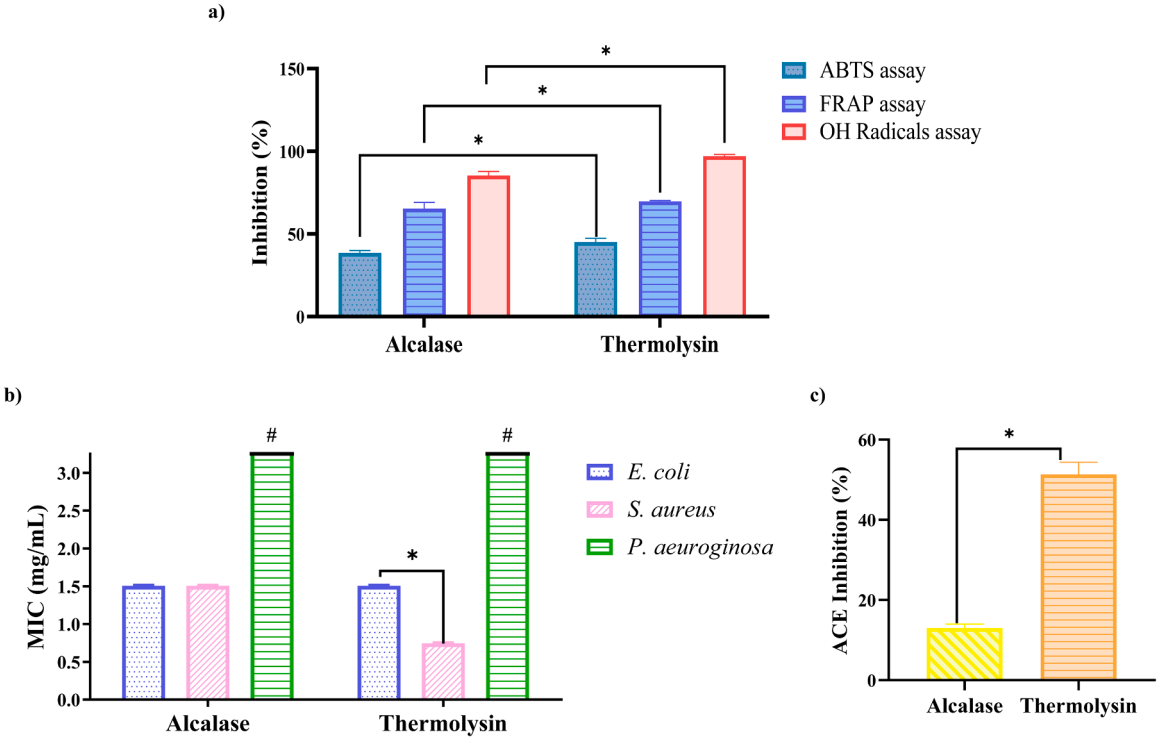


**Fig. 4.** Antioxidant, antibacterial, and antihypertensive activities of alcalase and thermolysin hydrolysates. # Indicates that the MIC value exceeds the maximum concentration tested (3 mg/mL). * Indicates statistically significant differences according to Student's t-test ($p$-value $< 0.05$). Controls: a) Antioxidant assays ABTS: Trolox 1 mg/mL (55 ± 3, %); FRAP: GSH 1 mg/mL (100 ± 7, %); Hydroxyl radicals: 1.5 mg/mL (98 ± 2, %). b) Antimicrobial assay: Streptomycin (MIC = 2.5 μg/mL). c) ACE inhibition: Captopril 50 μg/mL (100 ± 1, %).

mg/mL), highlighting its greater resistance [11,35]. In contrast, both hydrolysates inhibited the growth of *E. coli* and *S. aureus*, with MIC values of 1.5 mg/mL, except the thermolysin hydrolysate that exhibited a MIC of 0.75 mg/mL against *S. aureus*, ($p$-value $< 0.05$). This enhanced potential is consistent with prior studies reporting higher sensitivity of *S. aureus* to citrus-derived extracts [11]. Given the global rise in multidrug-resistant pathogens, the development of alternative antimicrobial agents is critical. In this context, antimicrobial peptides are promising candidates, typically characterized by low molecular weight and enrichment in F, W, R, and K, which facilitate membrane disruption [58]. Notably, thermolysin hydrolysates showed a higher abundance of phenylalanine residues, which may contribute to their superior antimicrobial activity. Additionally, naringenin-7-O-rutinoside was identified exclusively in the thermolysin hydrolysate (Table 5). Derivatives of naringenin have been reported to increase membrane permeability and reduce ATP production in bacteria, which may further explain the enhanced efficacy of this hydrolysate compared to alcalase [11]. Further to this, in light of prior studies employing SWE-EAE lime protein extracts hydrolyzed with alcalase or thermolysin, where MIC values ranged from 2.5 to 10 mg/mL, the notably lower MICs observed in the present work highlight the enhanced antimicrobial potential of the lemon PLE-derived hydrolysates.

In addition to antioxidant and antibacterial properties, the antihypertensive potential of lemon peel hydrolysates was also evaluated. As shown in Fig. 4**c**, ACE inhibitory activity reached 49 ± 3 % for the thermolysin hydrolysate and 13 ± 2 % for the alcalase one. The superior performance of the thermolysin hydrolysate may be partially attributed to the presence of the peptide LTPTSN (Table 4), previously identified as an ACE inhibitor *in Olea europaea* seed extracts [34] and FDAVGVK, identified as an antihypertensive agent in lime peel extract [27]. This suggests that specific peptide sequences generated during enzymatic hydrolysis may contribute to bioactivity.

Consistent with these findings, previous studies have reported low ACE inhibitory activity (10–30 %) in alcalase hydrolysates from lime peel, with improved inhibition when glucosidase-assisted extraction was applied [27]. In contrast, thermolysin hydrolysates have shown more variable but generally higher inhibition levels, ranging from 20 % to 65 %, depending on the extraction and hydrolysis conditions [4,27]. Therefore, the overall ACE inhibition of lemon peel hydrolysates was modest, indicating limited antihypertensive potential.

## 4. Conclusions

This study reports, for the first time, the recovery and characterization of protein extracts from lemon peels using an optimized PLE combined with UHPLC-ESI-QTOF-MS/MS method. The protocol, employing low ethanol content (18 % v/v), moderate temperature (110 °C), and short extraction time (7 min), demonstrated high efficiency and environmental compatibility, achieving a greenness score of 59 % (AGREEprep), supporting its potential for sustainable biorefinery applications. These optimized conditions provide a solid foundation for future process modeling and industrial scale-up initiatives. Then, the enzymatic hydrolysis with alcalase and thermolysin of the optimized PLE protein extracts yielded 58 peptides, including 39 derived from lemon proteins, identified by UHPLC-MS/MS, along with 19 polyphenols, 3 organic acids, 3 amino acids, and 2 purine nucleosides, indicating a diverse and multifunctional bioactive profile. Among the hydrolysates, thermolysin-derived extract exhibited the highest antioxidant, antimicrobial, and antihypertensive activities, likely due to the presence of specific peptides enriched in aromatic amino acids such as phenylalanine and tyrosine.

These findings highlight the potential of lemon peel as a valuable source of functional ingredients and demonstrate the effectiveness of combining green extraction technologies with bioactivity profiling for the development of natural products for food and nutraceutical applications.

## CRediT authorship contribution statement

**Esther Gómez-Mejía:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **María Concepción García:** Writing – review & editing, Visualization, Resources, Methodology. **María Castro-Puyana:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **María Luisa Marina:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2025.100218.

## Data availability

Data will be made available on request.

## References

[1] A.R.S. Mateus, A. Pena, A.S. Silva, Development of functional muffins enriched with lemon by-products as sources of bioactive compounds, Food Chem. Adv. 7 (2025) 100972, https://doi.org/10.1016/J.FOCHA.2025.100972.
[2] M.T. Spicher, K.M. Dressel, U. Schweiggert-Weisz, S. Gola, P. Eisner, Plant protein preferences in meat and dairy alternatives: an exploratory study of German consumers, Future Foods 11 (2025) 100595, https://doi.org/10.1016/J.FUFO.2025.100595.
[3] M.N. Nasrabadi, A. Sedaghat Doost, R. Mezzenga, Modification approaches of plant-based proteins to improve their techno-functionality and use in food products, Food Hydrocoll. 118 (2021) 106789, https://doi.org/10.1016/J.FOODHYD.2021.106789.
[4] A. Sánchez-Elvira, E. Hernández-Corroto, M.C. García, M. Castro-Puyana, M. L. Marina, Sustainable extraction of proteins from lime peels using ultrasound, deep eutectic solvents, and pressurized liquids, as a source of bioactive peptides, Food Chem. 458 (2024) 140139, https://doi.org/10.1016/J.FOODCHEM.2024.140139.
[5] United States Department of Agriculture /Foreign Agricultural Service, Citrus: World Markets and Trade, 2024. https://fas.usda.gov/data/citrus-world-markets-and-trade-07252024 (Accessed 20 August 2024).
[6] Food and Agriculture Organization (FAO), FAOSTAT, Crops and Livestock Products: Lemons and Limes, 2025. https://www.fao.org/faostat/en/#data/QCL/visualize (Accessed 10 July 2025).
[7] N.E. Wedamulla, M. Fan, Y.J. Choi, E.K. Kim, Citrus peel as a renewable bioresource: transforming waste to food additives, J. Funct. Foods 95 (2022), https://doi.org/10.1016/j.jff.2022.105163.
[8] A. Rosa, G.L. Petretto, M. Maldini, · B. Tirillini, M. Chessa, G. Pintore, G. Sarais, Chemical characterization, antioxidant and cytotoxic activity of hydroalcoholic extract from the albedo and flavedo of Citrus limon var. Pompia Camarda, J. Food Meas. Character. 17 (2023) 627–635, https://doi.org/10.1007/s11694-022-01659-w.

[9] S. Zahr, R. Zahr, R. El Hajj, M. Khalil, Phytochemistry and biological activities of citrus sinensis and citrus limon: an update, J. Herb. Med. 41 (2023) 100737, https://doi.org/10.1016/J.HERMED.2023.100737.
[10] G. Domínguez-Rodríguez, V.M. Amador-Luna, M. Castro-Puyana, E. Ibáñez, M. L. Marina, Sustainable strategies to obtain bioactive compounds from citrus peels by supercritical fluid extraction, ultrasound-assisted extraction, and natural deep eutectic solvents, Food Res. Int. 202 (2025) 115713, https://doi.org/10.1016/J.FOODRES.2025.115713.
[11] E. Gómez-Mejía, M.I. Dias, C. Pereira, T.C.S.P. Pires, J. Palá-Paúl, N. Rosales-Conrado, M.E. León-González, R. Calhelha, C.L. Roriz, A biorefinery approach for the simultaneous obtention of essential oils, organic acids and polyphenols from citrus peels: phytochemical characterization and bioactive potential, Food Chem. 486 (2025) 144641, https://doi.org/10.1016/J.FOODCHEM.2025.144641.
[12] M.G. Aguilar-Hernández, P. Sánchez-Bravo, F. Hernández, Á.A. Carbonell-Barrachina, J.J. Pastor-Pérez, P. Legua, L. Molina, Determination of the volatile profile of lemon peel oils as affected by Rootstock, Foods 9 (2020), https://doi.org/10.3390/foods9020241.
[13] A. Fuso, P. Viscusi, S. Larocca, F.S. Sangari, V. Lolli, A. Caligiani, Protease-assisted mild extraction of soluble fibre and protein from fruit by-products: a biorefinery perspective, Foods 12 (2023) 148, https://doi.org/10.3390/FOODS12010148.
[14] E. Espinosa, E. Rincón, R. Morcillo-Martín, L. Rabasco-Vílchez, A. Rodríguez, Orange peel waste biorefinery in multi-component cascade approach: polyphenolic compounds and nanocellulose for food packaging, Ind. Crops. Prod. 187 (2022) 115413, https://doi.org/10.1016/J.INDCROP.2022.115413.
[15] S. Arango-Manrique, T. Agudelo Patiño, L.G. Matallana Pérez, M. Ortiz-Sanchez, C. A. Cardona Alzate, Conceptual design and economic optimization of different valorization routes for orange peel waste: the application of the biorefinery concept for an integral use of raw material, Processes 12 (2024), https://doi.org/10.3390/pr12102298.
[16] E. Buket Karabiber, E. Yilmaz, Extraction and characterisation of lemon, orange and grapefruit seeds press cake proteins, Qual. Assur. Saf. Crops Foods 9 (2017) 357–367, https://doi.org/10.3920/QAS2016.0984.
[17] G. Capaldi, A. Binello, C. Aimone, S. Mantegna, G. Grillo, G. Cravotto, New trends in extraction-process intensification: hybrid and sequential green technologies, Ind. Crops. Prod. 209 (2024) 117906, https://doi.org/10.1016/j.indcrop.2023.117906.
[18] G. Alvarez-Rivera, M. Bueno, D. Ballesteros-Vivas, J.A. Mendiola, E. Ibañez, Pressurized liquid extraction. Liquid-Phase Extraction, Elsevier, 2019, pp. 375–398, https://doi.org/10.1016/B978-0-12-816911-7.00013-X.
[19] P. Alonso-Riaño, C. Ramos, E. Trigueros, S. Beltrán, M.T. Sanz, Study of subcritical water scale-up from laboratory to pilot system for brewer's spent grain valorization, Ind. Crops. Prod. 191 (2023) 115927, https://doi.org/10.1016/J.INDCROP.2022.115927.
[20] M.T. Fernández-Ponce, B.R. Parjikolaei, H.N. Lari, L. Casas, C. Mantell, E. J. Martínez de la Ossa, Pilot-plant scale extraction of phenolic compounds from mango leaves using different green techniques: kinetic and scale up study, Chem. Eng. J. 299 (2016) 420–430, https://doi.org/10.1016/J.CEJ.2016.04.046.
[21] C. Pronyk, G. Mazza, Design and scale-up of pressurized fluid extractors for food and bioproducts, J. Food Eng. 95 (2009) 215–226, https://doi.org/10.1016/J.JFOODENG.2009.06.002.
[22] M. Guzmán-Lorite, M.L. Marina, M.C. García, Pressurized liquids *vs.* high intensity focused ultrasounds for the extraction of proteins from a pomegranate seed waste, Innov. Food Sci. Emerg. Technol. 77 (2022), https://doi.org/10.1016/j.ifset.2022.102958.
[23] E. Hernández-Corroto, M. Plaza, M.L. Marina, M.C. García, Sustainable extraction of proteins and bioactive substances from pomegranate peel (Punica granatum L.) using pressurized liquids and deep eutectic solvents, Innov. Food Sci. Emerg. Technol. 60 (2020), https://doi.org/10.1016/j.ifset.2020.102314.
[24] S.O. Galván, E. González-García, M.L. Marina, M.C. García, Comparative study of factors affecting the recovery of proteins from malt rootlets using pressurized liquids and ultrasounds, Curr. Res. Food Sci. 5 (2022) 1777–1787, https://doi.org/10.1016/j.crfs.2022.10.009.
[25] AOAC International, AOAC: Official Methods of Analysis, 1980. https://archive.org/details/gov.law.aoac.methods.1980 (Accessed 27 July 2023).
[26] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – analytical greenness metric for sample preparation, TrAC Trends Anal. Chem. 149 (2022) 116553, https://doi.org/10.1016/j.trac.2022.116553.
[27] R.M. Palma-Manrique, M.C. García, M. Castro-Puyana, M.L. Marina, Simultaneous combination of subcritical water extraction and enzyme-assisted extraction for protein recovery from lime peels. Characterization of protein hydrolysates, Food Chem. 480 (2025), https://doi.org/10.1016/j.foodchem.2025.143910.
[28] E. Hernández-Corroto, M.L. Marina, M.C. García, Extraction and identification by high resolution mass spectrometry of bioactive substances in different extracts obtained from pomegranate peel, J. Chromatogr. A 1594 (2019) 82–92, https://doi.org/10.1016/J.CHROMA.2019.02.018.
[29] R. Vásquez-Villanueva, M.L. Marina, M.C. García, Revalorization of a peach (Prunus persica (L.) Batsch) byproduct: extraction and characterization of ACE-inhibitory peptides from peach stones, J. Funct. Foods 18 (2015) 137–146, https://doi.org/10.1016/j.jff.2015.06.056.
[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[31] N. Dabetić, V. Todorović, M. Panić, I.R. Redovniković, S. Šobajić, Impact of deep eutectic solvents on extraction of polyphenols from grape seeds and skin, Appl. Sci. 10 (2020) 4830, https://doi.org/10.3390/APP10144830.
[32] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428, https://doi.org/10.1021/AC60147A030/ASSET/AC60147A030.FP.PNG_V03.
[33] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying small molecules via high resolution mass spectrometry: communicating confidence, Environ. Sci. Technol. 48 (2014) 2097–2098, https://doi.org/10.1021/ES5002105.
[34] E. Hernández-Corroto, M.L. Marina, M.C. García, Multiple protective effect of peptides released from Olea europaea and Prunus persica seeds against oxidative damage and cancer cell proliferation, Food Res. Int. 106 (2018) 458–467, https://doi.org/10.1016/J.FOODRES.2018.01.015.
[35] E. Gómez-Mejía, L.H. Mikkelsen, N. Rosales-Conrado, M.E. León-González, Y. Madrid, A combined approach based on matrix solid-phase dispersion extraction assisted by titanium dioxide nanoparticles and liquid chromatography to determine polyphenols from grape residues, J. Chromatogr. A (2021) 1644, https://doi.org/10.1016/j.chroma.2021.462128.
[36] T. Lundstedt, E. Seifert, L. Abramo, B. Thelin, Å. Nyström, J. Pettersen, R. Bergman, Experimental design and optimization, Chemometr. Intell. Laborat. Syst. 42 (1998) 3–40, https://doi.org/10.1016/S0169-7439(98)00065-3.
[37] E. Gómez-Mejía, D. Vicente-Zurdo, N. Rosales-Conrado, M.E. León-González, Y. Madrid, Screening the extraction process of phenolic compounds from pressed grape seed residue: towards an integrated and sustainable management of viticultural waste, LWT 169 (2022) 113988, https://doi.org/10.1016/J.LWT.2022.113988.
[38] H.M. Charnock, G.J. Pickering, B.S. Kemp, The Maillard reaction in traditional method sparkling wine, Front. Microbiol. 13 (2022) 979866, https://doi.org/10.3389/FMICB.2022.979866/BIBTEX.
[39] L. El Hosry, V. Elias, V. Chamoun, M. Halawi, P. Cayot, A. Nehme, E. Bou-Maroun, Maillard reaction: mechanism, influencing parameters, advantages, disadvantages, and food industrial applications: a review, Foods. 14 (2025) 1881, https://doi.org/10.3390/FOODS14111881.
[40] J. Müller, Dumas or Kjeldahl for reference analysis? Comparison and considerations for nitrogen/protein analysis of food and feed A white Paper from FOSS, Hilleroed (2017).
[41] Q. Dai, Q. Hong, X. Zhu, P.S. Hornung, H. Wang, W. Fang, Y. Zhang, T. Beta, Formation of whey protein isolate–dextran conjugates by Maillard reaction with ethanol-water pretreatment, LWT 185 (2023) 115142, https://doi.org/10.1016/J.LWT.2023.115142.
[42] N.J. Kruger, The Bradford Method for Protein Quantitation, Springer Protocols Handbooks. Humana Press, Totowa, NJ, 2009, https://doi.org/10.1007/978-1-59745-198-7_4.
[43] M. Vergara-Barberán, Ó. Mompó-Roselló, M.J. Lerma-García, J.M. Herrero-Martínez, E.F. Simó-Alfonso, Enzyme-assisted extraction of proteins from Citrus fruits and prediction of their cultivar using protein profiles obtained by capillary gel electrophoresis, Food Control 72 (2017) 14–19, https://doi.org/10.1016/J.FOODCONT.2016.07.025.
[44] E. Fasoli, M. Colzani, G. Aldini, A. Citterio, P.G. Righetti, Lemon peel and limoncello liqueur: a proteomic duet, Biochim. Biophys. Acta 1834 (2013) 1484–1491, https://doi.org/10.1016/J.BBAPAP.2013.05.004.
[45] E. Gómez-Mejía, N. Rosales-Conrado, M.E. León-González, Y. Madrid, Citrus peels waste as a source of value-added compounds: extraction and quantification of bioactive polyphenols, Food Chem. 295 (2019) 289–299, https://doi.org/10.1016/j.foodchem.2019.05.136.
[46] P.L. Pasquet, D. Julien-David, M. Zhao, M. Villain-Gambier, D. Trébouet, Stability and preservation of phenolic compounds and related antioxidant capacity from agro-food matrix: effect of pH and atmosphere, Food Biosci. 57 (2024) 103586, https://doi.org/10.1016/J.FBIO.2024.103586.
[47] T. Brezo-Borjan, J. Švarc-Gajić, S. Morais, C. Delerue-Matos, F. Rodrigues, I. Lončarević, B. Pajin, Chemical and biological characterisation of orange (Citrus sinensis) peel extracts obtained by subcritical water, Processes 2023 11 (2023) 1766, https://doi.org/10.3390/PR11061766.
[48] A.M.B. Oliveira, J. Viganó, V.L. Sanches, M.A. Rostagno, J. Martínez, Extraction of potential bioactive compounds from industrial Tahiti lime (Citrus latifólia Tan.) by-product using pressurized liquids and ultrasound-assisted extraction, Food Res. Int. 157 (2022) 111381, https://doi.org/10.1016/J.FOODRES.2022.111381.
[49] J.R. Ayala, G. Montero, M.A. Coronado, C. García, M.A. Curiel-Alvarez, J.A. León, C.A. Sagaste, D.G. Montes, Characterization of orange peel waste and valorization to obtain reducing sugars, Molecules 26 (2021) 1348, https://doi.org/10.3390/MOLECULES26051348.
[50] R. Velmurugan, K. Muthukumar, Ultrasound-assisted alkaline pretreatment of sugarcane bagasse for fermentable sugar production: optimization through response surface methodology, Bioresour. Technol. 112 (2012) 293–299, https://doi.org/10.1016/J.BIORTECH.2012.01.168.
[51] UniProt, (n.d.). https://www.uniprot.org/(Accessed 22 July 2025).
[52] C.A. Ledesma-Escobar, F. Priego-Capote, M.D. Luque De Castro, Characterization of lemon (Citrus limon) polar extract by liquid chromatography–tandem mass spectrometry in high resolution mode, J. Mass Spectrometry 50 (2015) 1196–1205, https://doi.org/10.1002/JMS.3637.
[53] A. Brito, J.E. Ramirez, C. Areche, B. Sepúlveda, M.J. Simirgiotis, HPLC-UV-MS profiles of phenolic compounds and antioxidant activity of fruits from three citrus species consumed in Northern Chile, Molecules 19 (2014) 17400–17421, https://doi.org/10.3390/MOLECULES191117400.
[54] V.L. Sanches, T.A. Cunha, J. Viganó, L.M. de Souza Mesquita, L.H. Faccioli, M. C. Breitkreitz, M.A. Rostagno, Comprehensive analysis of phenolics compounds in citrus fruits peels by UPLC-PDA and UPLC-Q/TOF MS using a fused-core column,

Food Chem. X. 14 (2022) 100262, https://doi.org/10.1016/J.FOCHX.2022.100262.
[55] G. Domínguez-Rodríguez, V.M. Amador-Luna, K. Benešová, M. Pernica, F. Parada-Alfonso, E. Ibáñez, Biorefinery approach with green solvents for the valorization of Citrus reticulata leaves to obtain antioxidant and anticholinergic extracts, Food Chem. 456 (2024) 140034, https://doi.org/10.1016/J.FOODCHEM.2024.140034.
[56] L. Yu, M. Chen, J. Liu, X. Huang, W. He, Z. Qing, J. Zeng, Systematic detection and identification of bioactive ingredients from citrus aurantium L. var. Amara using HPLC-Q-TOF-MS combined with a screening method, Molecules 25 (2020) 357, https://doi.org/10.3390/MOLECULES25020357.
[57] X. Sun, C. Acquah, R.E. Aluko, C.C. Udenigwe, Considering food matrix and gastrointestinal effects in enhancing bioactive peptide absorption and bioavailability, J. Funct. Foods 64 (2020) 103680, https://doi.org/10.1016/J.JFF.2019.103680.
[58] M.G. Ciulla, F. Gelain, Structure–activity relationships of antibacterial peptides, Microb. Biotechnol. 16 (2023) 757–777, https://doi.org/10.1111/1751-7915.14213.