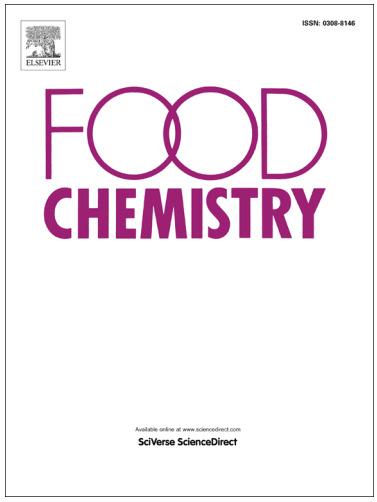

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Citrus peels waste as a source of value-added compounds: extraction and quantification of bioactive polyphenols

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ABSTRACT

A method combining solid-liquid extraction based on ethanolic aqueous solution, cLC-DAD and chemometrics, was performed to extract and quantify polyphenols from citrus peels. LC-MS/MS was also employed for chemical profiling.

The effect of extraction variables on the recovery was examined by experimental factorial design. Data were evaluated using multifactorial-ANOVA, response surface analysis and Principal Component Analysis.

trans-Ferulic and *p*-coumaric antioxidants were found in lower quantities (< 1.4 $mg \cdot g^{-1}$) in all peel extracts. Narangin flavonoid was also identified in all samples, while rutin flavonol was determined in the concentration range of 3.3-4.7 $mg \cdot g^{-1}$. The most abundant polyphenol in the extracts obtained from all evaluated citrus samples was the flavanone hesperidin (280-673 $mg \cdot g^{-1}$). Furthermore, peel extracts were evaluated in terms of total polyphenol and flavonoid content, total antioxidant activity and DPPH free radical scavenging.

The obtained results suggested that evaluated citrus peel by-products could be reused as a source of polyphenols and transformed into value-added products.

Keywords

Citrus waste, phenolic compounds, antioxidant activity, extraction, cLC-DAD analysis, LC-MS/MS confirmation, chemometrics

1. Introduction

In the last decades, world production of citrus fruit and fruit juice industry has experienced continuous growth. Thus, the European Fruit Juice Association reported that, globally, fruit juice and nectar consumption was 38.5 billion liters in 2015, being the EU the biggest consumption region, followed by North America (AIJN, 2016). The important productive activity of the juice sector generates huge amounts of waste materials every year, such as peels, membranes and seeds, representing citrus peel waste alone almost 50 % of the wet fruit mass (Sharma et al., 2017). These generated residues are mainly used as animal feed or direct discarded as waste to the environment, without proper processing (Garcia-Castello et al., 2015). The first solution is energy-costly on an industrial scale, and in both cases, certain environmental problems are caused. Many value-added compounds commercially important can be efficiently extracted from citrus peels, as well as be reused in several ways (Sharma et al., 2017). Hence, in sustainability terms, the main challenge of juice factories is to maximize the reuse and exploitation of the by-products generated during the production process. This necessarily involves the development of environmental friendly procedures able to recover and recycle the added-value compounds from citrus waste.

Citrus peels contain significant amounts of biologically active polyphenols, specifically phenolic acids and flavonoids, which have exhibited important antioxidant, anti-inflammatory, antiproliferative, anti-allergic, antiviral, anticarcinogenic, neuroprotective and antimicrobial properties (Oboh & Ademosun, 2012). Therefore, citrus peel waste from the juice sector could be consider as a valuable source of health promoting bioactive substances as phenolic compounds, with potential interest as ingredients in dietary supplements, raw materials in cosmetic, natural additives in food products and/or applications in the pharmaceutical and nutraceutical sectors (Rafiq et al., 2016).

Extraction is the initial step of value-added polyphenols recovery from citrus peel wastes. Recently, research and progress in developing effective extraction techniques for many industries has received particular attention due to the increase in energy prices, CO_2 emissions, among other environmental

problems (Sharma et al., 2017). Several methods based on conventional liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) by external-factor assistance (reflux, shaking, stirring, pressing or heating systems), microwave-assisted extraction (MAE), subcritical water or supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE) have already been used for the extraction of phenolic compounds. For example, García-Castello et al. (2015) extracted flavonoids such as neohesperidin, neoeritrocin, narirutin, naringin, hesperidin and tangeritin by SLE and UAE from grapefruit (*Citrus paradisi L.*) solid wastes, where UAE showed higher extraction yields with lower temperature and extraction time. Similar results were obtained by Wang et al. (2018) regarding tangeretin and nobiletin, where yields extracted from red orange peel using UAE were 1.5 times higher than those obtained using SLE. Other authors (Nayak et al., 2015) recovered gallic, chlorogenic, caffeic, *p*-coumaric and ferulic acids, rutin and catechin from *Citrus sinensis* peels using SLE, MAE, UAE and ASE. Therefore, it can be concluded that each one of the tested extraction techniques favored the extraction of specific types of phenolic compounds. Ferreira et al. (2018) combined SLE with reverse phase solid phase extraction (RP-SPE) enrichment method, for extracting polyphenols from *Citrus reticulata* Blanco peels, the main components (hesperidin, naringin, tangeritin, and rutin) accounting for nearly 86 % of the total polyphenols extracted. On the other hand, low power UAE showed greater extraction efficiency than MAE regarding hesperidin content extracted from mandarin peel (Nipornrama et al., 2018), UAE also being the adequate technique for simultaneous recovery of *p*-coumaric, caffeic and chlorogenic acids, and hesperidin from citrus waste (Papoutsis et al., 2018a).

However, most of these extraction techniques present certain limitations, such as compounds degradation due to high temperatures or high ultrasonic power applied, mass transfer resistance, long extraction times, large volume of solvents or health hazards. In some cases, the equipment required by the extraction methods is also difficult to operate and/or expensive (Garcia-Castello et al., 2015; Nayak et al., 2015; Nipornrama et al., 2018).

Additionally, the solvent nature plays an important role in achieving optimum recovery of bioactive polyphenols. Methanol, ethanol, acetone, and ethyl acetate are the most commonly used for the recovery of phenolic compounds from citrus peels (Papoutsis et al., 2016; Safdar et al., 2017). Despite their efficiency, cost, toxicity and safety concerns also exist over their use at industrial scale, water being the chosen solvent for high volume extraction. Therefore, scientific efforts should be focus on ways to improve the efficiency of aqueous extraction along with the development of safer, efficient, energy-saving and sustainable extraction processes for citrus waste reuse, which can also offer advantages to the food industry in terms of effectiveness, profitability, time and solvent consumption. These extraction methods could add value to the citrus processing industry, and set an example for achieving cleaner production of high-demanded phenolic compounds.

Therefore, the aim of the present study was to extract, identify and quantify bioactive polyphenols with potential interest in food, cosmetic and pharmaceutical industries from lemon, orange and clementine peels. For this purpose, an analytical methodology combining a simple, eco-friendly and cost effective extraction procedure with spectrophotometric mass spectrometric and liquid chromatographic (LC) methods were developed. Furthermore, to explore the obtained results and to determine the optimum extraction conditions, chemometric tools including experimental design, response surface analysis (RSA), multi-factorial analysis of variance (ANOVA), and principal component analysis (PCA) were applied. LC-MS/MS measurements were also employed for polyphenol chemical profiling.

2. Materials and Methods

2.1. Citrus samples

Citrus sinensis L. Osbeck (Navelate navel orange), *Citrus lemon* L. (lemon) and *Citrus x clementina* (clementine), all from Valencia region (Spain), were purchased from a local market at maturity. Fruits were carefully cleaned with distilled water to remove dirt, dust, microflora, and pesticide residue on the surface. Peels were separated by hand and air dried during three days maximum.

Then, they were cut into small pieces of a similar size (1 × 0.5 cm) using a stainless steel knife, and stored in hermetic glass containers under refrigeration until processing to prevent polyphenol degradation.

The moisture content of dried peel samples was obtained according to the AOAC method 20.013 (AOAC, 1980). It was determined as percentage (mean ± standard deviation, n=3) for lemon (21 % ± 1 %), clementine (38.8 % ± 0.6 %) and orange (14.5 % ± 0.2 %) peels.

2.2. Reagents, solvents and polyphenol standards

All chemicals and solvents were of analytical grade, and purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. Ethanol (EtOH), acetonitrile (MeCN) and methanol (MeOH) of gradient HPLC quality were provided by Scharlab (Barcelona, Spain). Dimethyl sulfoxide (DMSO, ≥99.9 %), 2N Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox, 97 %) and trifluoroacetic acid (TFA, 99 %), were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ammonium molybdate tetrahydrate, aluminium chloride 6-hydrate, sulphuric acid (95-98 %), sodium carbonate anhydrous, tri-sodium phosphate 12-hydrate, sodium hydroxide pellets and sodium nitrite were obtained from Panreac (Barcelona, Spain). For LC-MS/MS analysis, methanol, water and formic acid were of Optima™ LC/MS grade (Fisher Scientific, Fair Lawn, NJ, USA), and ethanol of BioUltra quality (≥99.8 %, Sigma-Aldrich).

Phenolic standards were obtained from Sigma-Aldrich: 3,4,5-trihydroxybenzoic acid monohydrate (gallic acid, ≥98.0 %); *trans*-4-hydroxycinnamic acid (*p*-coumaric acid, ≥98.0 %), *trans*-4-hydroxy-3-methoxycinnamic acid (*trans*-ferulic acid, 98 %); quercetin-3-rutinoside trihydrate (rutin); 3,3′,4′,5,5′,7-hexahydroxyflavone (myricetin); 3,4′,5-trihydroxy-*trans*-stilbene (resveratrol, ≥99 %); hesperetin 7-rhamnoglucoside (hesperidin, from European Pharmacopoeia); 4',5,7-trihydroxyflavanone 7-rhamnoglucoside (naringin, ≥95 %) and 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one (quercetin, ≥95 %).

The adequate amount of each polyphenol standard was dissolved into MeOH to obtain stock solutions at concentrations of 200 $mg \cdot L^{-1}$. Hesperidin and quercetin stock solutions were prepared using a 5% (v/v) DMSO aqueous solution and EtOH-H_2O mixture 80:20 (v/v), respectively. These solutions were stored in the dark at a temperature of 4 °C maximum for one month, with the exception of *trans*-ferulic acid, myricetin and hesperidin, which were stored at -80 ºC to prevent their degradation. Fresh working standard solutions were prepared daily, by diluting stock solutions as required.

2.3. Equipment

Chromatographic determination of polyphenols by cLC-DAD was carried out by an Agilent system Mod. 1100 Series (Agilent Technologies, Madrid, Spain), equipped with a G1376A binary capillary pump, a G1379A degasser, a G1315B diode array detector (500 nL, 10 mm pathlength), and the Agilent Chemstation software package for data collection and processing. An external stainless steel loop (10 µL) was positioned into a Rheodyne® injection valve. The reversed-phase separation was performed using a Synergi™ Fusion C18 capillary analytical column (150 mm × 0.3 mm I.D., 4 µm, Phenomenex, Torrance, CA, USA).

Confirmatory analyses by LC-MS/MS were performed on a Synergi™ C18 Fusion-RP 80 Å analytical column (150 x 3 mm I.D., 4 µm) from Phenomenex, employing a Shimadzu LC-MS-8030 triple quadrupole system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Nexera LC-30AD solvent delivery unit, a Nexera SIL-30AC autosampler with temperature-controlled tray, and a CTO-20AC column oven. Data acquisition and processing were made by using the LabSolutions LCMS software, also provided by Shimadzu. The instrument was operated in negative electrospray ionization (ESI) mode. Nitrogen was used as both nebulizing (1.5 $L \cdot min^{-1}$) and drying (15.0 $L \cdot min^{-1}$) gas. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 230 kPa in the collision cell, and the collision energy voltages applied

were in the range 10-55 eV (Table S1). ESI for ionization voltage was set at -4.5 kV. The interface current was fixed at 6.2 μA, and the detector voltage at 1.84 kV.

For absorbance measurements, a diode array HP8543 UV/Vis spectrophotometer (Agilent Technologies), equipped with the HP Chemstation software, was employed.

A Unicen centrifuge supplied by Ortoalresa (Madrid, Spain) was employed for obtaining the citrus peel extracts before the chromatographic analysis. For determination of peel moisture content, a P-Selecta oven with temperature control in the range 10-200 ºC from Panreac (Barcelona, Spain) was used. Extraction of polyphenols from the citrus peels was accomplished in an ultrasound bath purchased from P-Selecta, a WX vortex mixer and a magnetic stirrer with ceramic heating plate model HSC, both from VELP Scientifica (Usmate, MB, Italy).

2.4. Polyphenol extraction from citrus peels waste

The effect of several extraction conditions over solid-liquid extraction was evaluated by means of a two-stage cLC-DAD study. The first stage was conducted to establish the factors and their experimental relevance on phenolic extraction efficiency. In this study, the factors were selected according to the results obtained in preliminary assays, and samples were prepared in triplicate in all studied conditions. Thus, an amount of 0.30 g of air dried peels was added to 50 mL of Milli-Q water or EtOH-H_2O (20:80 v/v). The resulting mixtures were extracted during 15 min at 25 ºC. Vortex, ultrasound and magnetic stirring at 2 rpm were evaluated as extraction methods. When magnetic stirring was done, temperature was also fixed at 62 or 90 ºC. The obtained extracts were centrifuged at 4200 rpm during 10 min, before chromatographic analysis

The second stage was aimed to determine the best extraction conditions by analyzing the most relevant factors through experimental design and response surface analysis (RSA). A factorial (lemon and clementine peel) or a multi-level factorial (orange peel) designs were employed for each extraction system, fixing the use of magnetic stirring as extraction technique. The three independent experimental factors considered were: extraction time (X_1; 10-15 min), EtOH-H_2O proportion of

extraction solvent (X_2; from 20:80 to 40:60 v/v), and extraction temperature (X_3; from 62 to 90 ºC). The response variables used were extracted contents of *p*-coumaric acid, *trans*-ferulic acid, rutin and hesperidin, expressed as µg per gram of dried peel. The experiments were performed in random order in one block. In the case of orange peel, three levels (−1, 0, +1) were investigated for both EtOH-H_2O proportion of extraction solvent and extraction temperature, while two levels (−1, +1) were considered for extraction time. Thus, a total of 21 runs, 18 factorial points and three replicates of the center point, were accomplished (Table S2). Lemon and clementine peels were evaluated by means of a factorial design at two levels (−1, +1), also including three replicates corresponding to the central zone of the studied domain (11 runs) (Table S3-S4).

Response surface methodology (RSM) was applied to obtain the optimal conditions for maximum extraction of each phenolic compound and studied responses were fitted to a polynomial equation. To determine the experimental factor combination, which simultaneously optimize the experimental responses, multiple response analyses (MRA) were carried out. Optimal conditions for the extraction were selected by using a weighted desirability function (targeted to find a compromise that allows maximizing the extraction of each analyzed polyphenol). Verification of the validity and adequacy of the predictive extraction models was checked with the optimal conditions of extraction (three replicates) comparing predictions with observed values using a two sided t-test ($\alpha = 0.05$).

For the analysis of citrus peel extracts by LC-MS/MS, 0.30 g of peel waste was extracted by applying optimal extraction conditions, deduced from RSM and MRA. Polyphenols from clementine and lemon peels were extracted by magnetic stirring at 2 rpm and 90 ºC for 15 min, using 50 mL of a mixture EtOH-H_2O with 20 % or 40 % (v) ethanol for clementine and lemon, respectively. Orange peels were treated under similar conditions during 10 min, using a mixture EtOH-H_2O 40:60 (v/v) as extraction solvent. Once cooled, extracts were centrifuged at 4200 rpm during 10 min.

Prior to LC–MS/MS analysis all sample extracts were filtered by using PTFE syringe filters (0.22 µm pore size) from Membrane Solutions (Kent, WA, USA).

2.5. Total polyphenol content (TPC)

Total phenolic content was measured according to the Folin-Ciocalteu colorimetric method described by Vijayalaxmi *et al*. (2015), but including slight modifications. Briefly, citrus peel samples (0.30 g) were weighted by triplicate and mixed with 50 mL of EtOH-H_2O solution (20:80 v/v for clementine peels, and 40:60 v/v for both lemon and orange peels). Extraction was accomplished by magnetic stirring during 15 min at 90 ºC (10 min for orange peels). The obtained extracts were cooled at 25 ºC and centrifuged at 4200 rpm for 10 min. To aliquots of 500 µL (orange peel) or 200 µL (lemon and clementine peels) of supernatants, 50-70 µL of the reactive Folin-Ciocalteu, 40-60 µL of Na_2CO_3 7.5 % (w) and Milli-Q water to a volume of 10 mL were consecutively added. Afterwards, the absorbance was measured at 720 nm with a UV-Vis spectrophotometer.

A similar procedure was carried out for preparing gallic acid standard solutions, and the calibration curve (n=5) was obtained within 0-40 µM concentration range. TPC was estimated as gallic acid equivalents/mL (mg GAE·mL^{-1}), and recalculated as mg of gallic acid equivalents per gram of dried peel (mg GAE·g^{-1}).

2.6. Total flavonoid content (TFC)

Flavonoids were determined by spectrophotometry using a slightly modified method reported by Vijayalaxmi *et al*. (2015). Citrus peel extracts were prepared in triplicate as described in Section 2.5. The reagents were added to a 10 mL volumetric flask in the following order: 2 mL of Milli-Q water, 0.15 mL of $NaNO_2$ 5 % (w) aqueous solution and 500 µL of citrus peel extracts. After 5 min, 0.15 mL of $AlCl_3$ 10 % (w) solution was incorporated. After reacting five more minutes, 1 mL of 1 M NaOH was added. The reaction solution was mixed manually and it was kept at room temperature for 15 min. Then, Milli-Q water was put to a final volume of 10 mL. The absorbance of the reaction mixture was subsequently measured at 415 nm. Quantification was done by employing

quercetin polyphenol as standard. Calibration graphs were obtained in the concentration range 0-40 μM, by preparing quercetin standard solutions in the same way as sample solutions. TFC was expressed as mg of quercetin equivalents per gram of sample dry weight (mg QE·g^{-1}).

2.7. Total antioxidant activity (TAA)

The experimental procedure was carried out as proposed by Shrikanta *et al*. (2015) by including some modifications. Determination of TAA was based on the reduction of Mo (VI) to Mo (V) by the citrus peel extract, followed by the formation, at acidic pH, of a green phosphate-Mo (V) complex.

Amounts of 0.1 g of citrus peel were weighted by triplicate, homogenized in mortar and pestle with 25 mL of EtOH-H_2O (80:20 v/v). The mixture was kept in a water bath at 60 °C during 30 min. These crude extracts were cooled, and they were maintained at room temperature until analysis. Previously, a reagent solution was prepared by mixing equal volumes of 1.8 mM sulphuric acid, 84 mM Na_3PO_4, and 12 mM $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$. To a volume of 100 μL of crude extracts, 6 mL of the prepared reagent solution were added. The resulting solutions were incubated in a water bath during 90 min at temperature of 95 °C. Once cooled down at room temperature, Milli-Q water was incorporated until a volume of 10 mL. The absorbance of these final solutions was measured with a spectrophotometer at a wavelength of 695 nm.

Gallic acid was used as a standard polyphenol to build the calibration curves (n=5) in the concentration range 0-15 μM. Calibration standard solutions were prepared in a similar way that crude extracts. Finally, total antioxidant activity was established as the gallic acid equivalents per gram of dried citrus peel (mg GAE·g^{-1}).

2.8. DPPH free radical scavenging assay

Free radical scavenging ability of citrus peel extracts against DPPH (1,1-diphenyl–2 picrylhydrazyl) was evaluated as described by Villaño et al. (2007), applying slight modifications. Prior to

spectrophotometric determination, samples (0.30 g) were weighted by triplicate and mixed with 50 mL of an EtOH-H_2O solution (20:80 v/v for clementine peels, and 40:60 v/v for both lemon and orange peels). Extraction was made by magnetic stirring at 90 ºC for 15 min (10 min for orange peels). Once cooled, peel extracts were centrifuged at 4200 rpm during 10 min.

For determining the antioxidant effect of citrus extracts on DPPH radical, five different solutions were prepared adding 5 mL of DPPH 0.28 mM methanolic solution and 1.0-5.0 mL of citrus extracts to a final volume of 10 mL. A DPPH control was also prepared with 5 mL of DPPH 0.28 mM methanolic solution and the corresponding volume (1.0-5.0 mL) of EtOH:H_2O extraction solvent, adding water to a final volume of 10 mL. Additionally, a blind control was prepared containing the sample extract and MeOH pure solvent instead of DPPH solution. Absorbance was recorded at 515 nm at different time intervals until the reaction reached an equilibrium (40 min for orange and lemon peels and 60 min for clementine peel), keeping the solutions at room temperature in the dark throughout the analysis time. It is necessary to define steady-state time to ensure a constant absorbance value.

A similar procedure was carried out for trolox standard solution within 2.5-150 μg concentration range.

The DPPH free radical scavenging ability was subsequently calculated as DPPH remaining percentage, according to the following equation:

$$\%\ DPPH\ remaining = \left(\frac{A_f}{A_0}\right) x\ 100$$

where A_0 is the absorbance value of DPPH control solution at 0 min and A_f is the absorbance of DPPH solution after the addition of the sample at steady state (40 min for orange and lemon peels or 60 min for clementine peel). Concentrations of peel extract or trolox standard were plotted against DPPH remaining percentages at steady state, so as to obtain the half maximal effective concentration (EC50), defined as the antioxidant amount needed to reduce 50% of the initial DPPH• concentration. Finally, DPPH scavenging activity was expressed in terms of mg of trolox

equivalents per gram of dried peel (mg·TE·g^{-1} DW), and mg of extract per gram of dried peel (mg extract·g^{-1} DW), both at EC_{50} value.

2.9. cLC-DAD analysis of individual polyphenols

Chromatographic separation was performed by a linear gradient combining solvent A (acetonitrile) and solvent B (aqueous solution 0.1% (v) trifluoroacetic acid at pH 3.2). The elution program consisted of: 12% A (3 min), then a linear increase to 15% A during 4 min, and finally an isocratic step at 15% A until the end of the chromatogram. Flow rate was maintained at 10 µL·min^{-1}, and the system was equilibrated between runs for 15 min using the start mobile phase composition.

As a result of a previous study (León-González et al., 2018), chromatographic analysis was carried out at room temperature using simultaneous monitoring at the following wavelengths: 220, 260, 292, 310 and 365 nm. Quantitative analyses were performed at 260 nm for rutin, 292 nm for gallic acid, naringin and hesperidin, 310 nm for *p*-coumaric acid, *trans*-ferulic acid and resveratrol, and 365 nm for myricetin. Large volumes (10 µL) of both sample extracts and standard solutions were injected for maximum sensitivity. In order to achieve the analyte on-column focusing, all injection solutions were prepared in an aqueous solution at pH 3.2 containing 0.1% (v) TFA and 1% (v) of acetonitrile.

Optimization of chromatographic conditions were made by using standard mixtures of polyphenols, prepared at concentrations of 50 µg·L^{-1}.

For the analysis of citrus peels, volumes of 10 or 20 µL from the sample extracts obtained as described in Section 2.4, were diluted to 5 mL with the focusing injection solution. Polyphenols were identified by matching the retention time and their spectral characteristics against those of standards, and quantification was made according to linear calibration curves of standard compounds.

Method performance was assessed for standard solutions considering the linearity range, limits of detection (LOD) and quantitation (LOQ), and precision. Validation was accomplished under

optimum experimental conditions mentioned above, following the recommendations collected in the ICH document on validation methodology (ICH, 2005), as well as the procedures described by Rambla-Alegre *et al*. (2012).

Linear ranges (n=8) were set at concentrations between 18 and 100 μg·L^{-1} for gallic acid, 5-100 μg·L^{-1} for *p*-coumaric acid, 10-100 μg·L^{-1} for *trans*-ferulic acid, rutin, naringin and resveratrol, 100-500 μg·L^{-1} for hesperidin and 75-500 μg·L^{-1} for myricetin. Chromatographic peak areas were analyzed by least squares linear regression, and linearity was evaluated in terms of R^2 values. Calibration curve equations were used for quantifying the studied polyphenols in citrus peel extracts. However, due to the high content of hesperidin in all analyzed samples, external calibration was also performed in the concentration range from 0.10 to 9.0 mg·L^{-1}.

LODs and LOQs were calculated as 3.3 SD/S and 10 SD/S, respectively. The standard deviation (SD) of the response was established on the basis of the residual SD of a regression curve obtained around LOD concentrations, being the S coefficient the slope of this calibration curve.

Precision was estimated from phenolic standard solutions at two different concentrations: 150 and 450 μg·L^{-1} for both hesperidin and myricetin, and 25 and 85 μg·L^{-1} for the rest of studied polyphenols. Intra-day variation (n=3) was evaluated by analyzing three standard solutions on the same day. Inter-day precision was similarly calculated from three successive days, by performing three injections per day (N=9). Relative standard deviation (RSD, %) was taken as a measure of repeatability and intermediate precision, being calculated for retention factor (*k*) and peak areas of each analyte.

2.10. Determination of phenolic compounds by LC–MS/MS

Analyses of citrus peel extracts by LC–MS/MS were made at room temperature, using a mixture of 0.2% (v) formic acid aqueous solution (solvent A) and methanol (solvent B) as mobile phase. Gradient elution was performed using the following ratios: 5% solvent B holding for 0.1 min, linear increase to 40% B within 25 min, and to 70% B within another 10 min. This condition was held for

2 min, then changed to the initial conditions (5% B) within 1 min and equilibrated for 2 min. The flow rate was 0.50 mL·min^{-1}, and the injection volume was set at 20 µL.

For preparation of injection solutions, aliquots of 40 µL from peel extracts (see Section 2.4) were mixed with 2.5 mL of methanol containing 0.2% (v) formic acid, and finally diluted to 5 mL with LC/MS grade water.

Full-scan mass spectra and MS/MS spectra were acquired in order to obtain the maximum number of available transitions for each analyte. The best results were obtained with ESI in the negative ionization mode, using the $[M-H]^-$ as precursor ion. Detection was carried out in multiple reactions monitoring (MRM) mode, with a dwell time of 100 ms, by monitoring three selective transitions for each parent compound. The most sensitive transition was selected for quantification whereas the other ones were taken as confirmation transitions. MS/MS parameters for every MRM chromatogram are shown in Table S1. External calibration (n=5) was performed in the concentration range of 10-80 µg·L^{-1} for *p*-coumaric acid, resveratrol and hesperidin, 20-100 µg·L^{-1} for both rutin and naringin, 20-80 µg·L^{-1} for *trans*-ferulic acid, 30-130 µg·L^{-1} for gallic acid, and 5-50 µg·L^{-1} for quercetin and myricetin. The correlation coefficients (R^2) of the calibration curves were between 0.9847 and 0.9984 for all standard compounds.

2.11. Statistical analysis

Data were statistically analyzed using multi-factorial analysis of variance (ANOVA) to determine the effect of independent variables (time, extraction solvent and temperature) on every phenolic compounds. Experimental data of each factorial design were submitted to response surface analysis (RSA) for establishing a mathematical model and obtaining the optimum conditions for simultaneous and maximum recovery of bioactive polyphenols. Data from extraction assays were also explored and modeled using principal component analysis (PCA). On the other hand, the mean values of different groups were compared using one-way ANOVA. The software package

Statgraphics Centurion 18 (Statgraphics Technologies, Inc., Warrenton, VA, USA) was employed for the statistical data evaluation.

3. Results and Discussion

3.1. Optimization and validation of cLC-DAD separation

Over the years many chromatographic methods have been developed for determination of polyphenolic compounds in diverse and different matrix. Thus, reversed phase LC techniques coupled with spectrophotometry multiple wavelength or mass spectrometry detectors represents the most popular and highly applicable technologies for separation, identification and quantification of bioactive polyphenols (Nazck & Shahidi, 2004). Although chromatography-mass spectrometry and multiple mass spectrometry are the more effective tool for structural characterization of polyphenols. However, diode array detection (DAD) carries out an appropriate way of polyphenolic identification, not only because of their lower cost but also because of their versatility and reliable results (Escarpa & González, 2001).

In this work, a cLC-DAD analytical method was developed, optimized and validated for the determination of gallic acid, *p*-coumaric acid, *trans*-ferulic acid, naringin, hesperidin, rutin, myricetin, resveratrol and quercetin. All of them have been previously reported as main polyphenols found in fruit. Naringin was the predominant flavonoid found in pummelo peel varieties (Xi et al., 2014) and gallic acid, *p*-coumaric acid and resveratrol were extracted from several underutilized fruits (Shrikanta et al., 2015). Rutin, hesperidin and *trans*-ferulic acid were also obtained from *Citrus sinensis* peels (Nayak et al., 2015), while both quercetin and kaempferol from kinnow mandarin peel extracts (Safdar et al., 2017).

In order to achieve optimal chromatographic resolution in the minimum analysis time, different separation conditions were tested by injecting standard solutions of target polyphenols. Due to the wide range of polarity of phenolic compounds, several elution gradients were evaluated on the basis of LC separation proposed by León-González et al. (2018). Thus, simple linear gradients with

MeCN-TFA aqueous solution based mobile phase were considered. For focusing purposes on the capillary column head and therefore, maximum sensitivity, composition of the injection solution was also optimized. By applying the optimal chromatographic conditions detailed in Section 2.9, separation was achieved in about 30 min.

Calibration was performed using analyte standard solutions prepared in a pH 3.2 aqueous solution containing 0.1% (v) TFA and 1% (v) of acetonitrile. Linearity and correlation coefficients of calibration curves (n=8) were determined by external calibration using peak area values as response. As can be observed in Table 1, good linearity was observed for all analytes at concentrations within the tested intervals, with determination coefficients (R^2) higher than 0.9918 in all cases. On the other hand, acceptable detection limits between 1.2 for *p*-coumaric and 22 $\mu g \cdot L^{-1}$ for myricetin were estimated. Repeatability and intermediate precision were evaluated calculating the relative standard deviation (RSD, %) by means of retention factor (*k*) and peak area, for each compound at two different concentrations. Intra-day precision was estimated from three consecutive injections on the same day, and inter-day variation from three successive days (three injections per day). Good intra-day variations were obtained for area and retention factor. RSD values between 1.5-7.4% were estimated for inter-day precision of retention factor and between 3.0 and 13% for peak areas, showing a satisfactory performance and repeatability of the chromatographic method.

3.2. Chromatographic polyphenol profile of the citrus peel extracts: effect of extraction conditions and statistical analyses

Individual polyphenol determination in the citrus peel extracts comprised a comprehensive optimization of sample extraction procedure and chromatographic conditions. Once optimized the cLC-DAD analytical method, several studies were intended to establish the best extraction conditions for polyphenol recovery.

Considering the preliminary experiments performed in our research group (León-González et al., 2018), different factors such as type of extraction (vortex, ultrasound and magnetic stirring),

extraction solvent (water or EtOH-H_2O 20:80 v/v) and temperature (25 ºC and 90 ºC) were firstly evaluated. On the basis of the preliminary extraction results (Figure S1), the extraction time was set at 15 min, the volume of extraction solvent at 50 mL and the amount of extracted sample was fixed at a mass of 0.30 g. Extraction temperature and composition solvent were the main factors affecting the extracted amount of each polyphenol. In fact, high extraction efficiency was obtained, in all experiments, using magnetic stirring at 2 rpm, temperatures higher than 25 ºC and EtOH-H_2O mixtures as extraction solvent. Consequently, design of experiments was applied to facilitate the optimization of the conditions affecting sample extraction.

3.2.1. Design of experiments (DOE) and response surface methodology (RSM)

Both DOE and RSM approaches were used effectively to establish the best extraction conditions from a reduced set of experiments, as well as to find out the relationships among the variables optimized.

Following the procedure described in Section 2.4, orange peel was deeply examined as reference matrix while for both lemon and clementine simpler experimental designs (only 11 runs) were planned. All citrus peel extracts were analyzed by cLC-DAD. Gallic acid, myricetin, resveratrol and naringin were not detected under all the experimental conditions employed for polyphenols extraction. Consequently, extracted contents of *p*-coumaric acid, *trans*-ferulic acid, rutin and hesperidin, expressed as μg per gram of dried peel, were established as response variables and fitted to polynomial models according to the following equations:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \text{ (lemon and clementine)}$$

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \text{ (orange)}$$

where Y is the predicted response variable; β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients of X_1 (extraction time), X_2 (EtOH-H_2O proportion of extraction solvent) and X_3 (temperature); β_{11}, β_{22}, *and* β_{33} are the squared coefficients of X_1, X_2 and X_3, and β_{12}, β_{13} and β_{23} are the interaction coefficients of X_1, X_2 and X_3, respectively.

The coefficient of multiple determination obtained (R^2) showed that, in general, the mathematical models developed to explain the influence of these factors on extraction efficiency provided an adequate explanation of the extraction results (Table 2). In the studied domain, the quadratic terms had not a significant influence in the extraction of *p*-coumaric, *trans*-ferulic, rutin and hesperidin from orange peels. The interaction between factors had a significant influence, at a confidence level of 95%, in the extraction of *p*-coumaric acid from both lemon and orange peels, rutin from lemon, and hesperidin from orange peels. As can be observed in Table 2, the models showed that, generally, temperature had a significant influence in the extraction procedure. However, EtOH-H_2O ratio was the only factor affecting the amount of hesperidin extracted from lemon peel, while none of the studied factors significantly affected the extraction of *trans*-ferulic acid from orange and clementine peels. By contrast, all factors had a significant effect on the extraction of hesperidin from clementine, and *p*-coumaric acid from orange peels.

In order to easily visualize the most important effects and their interactions on the extracted contents ($mg \cdot g^{-1}$), estimated normalized response surfaces were obtained for each polyphenol through their respective equations. These three-dimensional graphs showed more or less distorted planes with negligible or not factor interactions. As example, Fig. 1 shows the estimated normalized response surfaces obtained for the extracted content of *trans*-ferulic and *p*-coumaric acid from lemon and orange peels, respectively. In general, the highest response was found at high temperature (90 ºC) with the increase in the EtOH ratio. Extraction time had an important factor in the extraction of *p*-coumaric acid from orange peel (Fig. 1b), moreover time-extraction solvent interaction produced a significant effect.

To find the best conditions for individual polyphenol extraction from each matrix, maximum extracted content was established as the optimization criteria (Table 2). In the case of both orange and lemon peels, the optimum extraction conditions for each polyphenol were the same. Regarding clementine peels, the experimental conditions predicted by the models for the maximum extraction agreed only for *p*-coumaric acid, *trans*-ferulic acid and rutin. Consequently, multiple response

analysis (MRA) was carried out. This chemometric procedure allowed us to determine the combination of factor levels, which simultaneously optimize the studied responses and therefore, maximize the desirability function over the selected region. Optimization criterion for MRA was as well maximum recovery of each polyphenol, and a good desirability function value, around 0.75, was achieved (Fig. 1c).

To check the reliability of the predictive extraction models, predictions (Table 2) were compared with the observed values following the procedure described in Section 2.4. The statistical analysis showed that there were no significant differences between experimental and predicted results at the confidence level of 95% for all polyphenols. Accordingly, the proposed models allow establishing specific conditions for the extraction of a single or some polyphenols, also predicting the extracted amounts under different experimental conditions. In order to obtain the maximum extracted contents for all studied polyphenols, the optimum conditions selected for extraction from lemon peels were: 15 min for extraction time, EtOH-H_2O 40:60 (v/v) as extraction solvent, and 90 ºC as extraction temperature. In the case of orange peels 10 min, EtOH-H_2O 40:60 (v/v) and 90 ºC were selected. And finally, for clementine peels, MRA optimum conditions were established at 15 min, EtOH-H_2O 20:80 (v/v) and 90 ºC. Therefore, it seems to be that higher temperature may facilitate higher recovery of polyphenols by: i) affecting the physical properties of the extraction solvent and by extension of magnetic stirring effects, ii) enhancing the solubility of phenolic compounds which increases mass transfer rate from the citrus peel matrix into the solvent. High ratios of ethanol were also needed for polyphenol extraction from lemon and orange peels, but only 10 min were enough to extract the maximum content of polyphenols from orange peels. It is worth mentioning that the ethanol-water proportion of extraction solvent is not a significant factor for the recovery of rutin and *trans*-ferulic from orange and clementine, and *p*-coumaric acid from lemon and clementine peels (Table 2). Thus, the ethanol ratio could be decreased for the extraction of a single and specific polyphenol.

Compared to the polyphenol conventional solvent extraction (CSE) conditions deduced by other authors (Table S5), the proposed extraction method uses lower ratios of EtOH (maximum 40 %, v), very low extraction times (10-15 min) and low sample-to-solvent ratios (0.3 g/50 mL). The studies reported in the literature, frequently employed extraction solvents like pure acetone and aqueous solutions containing between 50% and 85% (v) of EtOH, or more than 50 % (v) of acetone (ACTN), combined with extraction times from 40 to 413 min or even 72 h. In this study, the optimum extraction temperature for obtaining the maximum extraction efficiency for all studied polyphenols was fixed at 90 ºC, which is higher than the temperatures employed in other CSE studies (34-60 ºC). This high temperature could be compensated by the rapidity and efficiency in the extraction process, although, depending on the matrix and target polyphenols, it could be decreased in some extractions conditions down to 62 ºC (Tables S2-S4). Other extraction techniques employed such as accelerated assisted solvent extraction (ASE), microwave assisted extraction (MAE), maceration extraction method (MEM) and ultrasound assisted extraction (UAE) use high ratios (> 50 %, v) of toxic organic solvents (methanol, acetone, ethyl acetate), extraction times in the range 90-240 s (MAE), 15-70 min (UAE), 40 min-20 h (MEM) or 23 min (ASE), and temperatures from 23 ºC (UAE) to 120 ºC (ASE) (Table S5). This fact again evidences the advantage of the proposed extraction method regarding the rapidity, simplicity of operation and/or equipment, and the use of aqueous solutions containing low ratios of ethanol, 20-40% (v), as the extraction solvent. Ethanol is an eco-friendly solvent and it could be purchased as a product from biorefinery. Furthermore, it could probably be recovered at the end of the extraction process at an industrial scale, also decreasing economic costs.

Other studies have employed water at temperatures around 50 ºC for the extraction of polyphenols from *Citrus limon* pomace (Papoutsis et al., 2018a) and lemon by-products (Papoutsis et al., 2018b) using UAE techniques. However, longer times were required for the extraction of rutin (35 min) from lemon by-products (Papoutsis et al., 2018b), and for *p*-coumaric acid (60 min) and hesperidin (40 min), both from citrus pomace (Papoutsis et al., 2018a). In addition, a sample-to-solvent ratio of

1 g/100 mL was needed, limiting the applicability of pure water as a solvent (Papoutsis et al., 2018a, 2018b).

With respect to polyphenol amounts, as included in Table 2, the maximum content of *p*-coumaric, *trans*-ferulic acid and hesperidin were achieved from clementine, and rutin from orange peels, although extracted content of rutin was similar to clementine one. *p*-Coumaric acid was extracted at low concentration levels (around 0.100 $mg \cdot g^{-1}$), while hesperidin extraction contents were three order of magnitude greater. Consequently, it could be concluded that clementine peel extracts are an important and abundant source of high value-added polyphenols, especially bioactive hesperidin.

It is important to remark that the optimum extraction conditions described by other authors in the literature have mostly been deduced from experimental designs based on total parameters such as total polyphenol content (TPC) or antioxidant activities (Table S5), and not from individual polyphenol contents. As referred in Section 3.3, total parameters measured by spectrophotometric methods are useful only for an approximate estimation of the extract properties. Thus, compared to the quantities reported for some individual polyphenols (Table S5), the extracted amounts by the present method (*trans* ferulic acid 0.29-1.38 $mg \cdot g^{-1}$; rutin 3.3-4.7 $mg \cdot g^{-1}$; hesperidin 280-673 $mg \cdot g^{-1}$) were considerably higher. Liew et al. (2018) extracted ferulic acid by CSE from *Citrus sinensis* peels in the range 0.7-0.9 $mg \cdot g^{-1}$, and Safdar et al. (2017) did it by UAE from *Citrus reticulate L*. peel at 0.1 $mg \cdot g^{-1}$ levels. Extracted amount of rutin by ASE from *Citrus sinensis* peels was 1.2 $mg \cdot g^{-1}$ (Nayak et al., 2015), while it was 3.2 $mg \cdot g^{-1}$ from lemon by-products by UAE requiring an extraction time of 35 min and a sample-to-solvent ratio of 1 g/100 mL (Papoutsis et al., 2018b). Regarding hesperidin, Nipornram et al. (2018) reported extracted amounts of 64.4 and 61.5 $mg \cdot g^{-1}$ from *Citrus reticulata* Blanco peel by UAE and MEM, respectively, using acetone-water (80:20 v/v) and 40 min as extraction time. The hesperidin yield obtained from *Citrus limon L* pomace was 7.84 $mg \cdot g^{-1}$, using UAE with pure water at 50 ºC during 40 min and a sample-to-solvent ratio of 1 g/100 mL. Therefore, the high quantities extracted using the proposed method prove that the sum of individual phenolic content extracted (Table S5) is probably the highest with regard to the one

reported in similar extraction studies from citrus waste, even though using the lowest sample amount.

3.2.2. Multi-factorial ANOVA

Chromatographic data were also analyzed using multi-factorial analysis of variance to confirm the effect of independent variables (time, solvent and temperature) on individual extraction of phenolic compounds, as well as to evaluate the influence of the nature of citrus peels. The obtained results mostly confirmed what RSM and MRA provided. As can be seen in Fig. 2, multi-factorial ANOVA test showed significant differences in polyphenol content for the different studied samples, nature of extraction solvent and temperature. Clementine peel yield the highest content of hesperidin, *trans*-ferulic and *p*-coumaric acid, while the amount of rutin extracted from all citrus peels was quite similar. In fact, the nature of the citrus peel had not shown a statistically significant effect at a confidence level of 95 % on rutin extraction. By contrast, the effect of the matrix was significantly influential for hesperidin extraction. Thus, the high hesperidin contents were achieved from clementine extracts, followed by orange and, finally, lemon peels.

p-Coumaric and *trans*-ferulic acids showed analogous behaviors, being extraction conditioned by the nature of the sample and temperature. Orange peel provided the lowest contents of both polyphenols, and the highest extracted amounts were obtained employing an extraction temperature of 90 ºC. In the case of hesperidin, the extraction solvent had a significant effect, which was confirmed by RSM in all studied peels. Additionally, hesperidin extraction was promoted using high ratios of ethanol in the extraction solvent.

3.3. Estimation of total phenolic contents and antioxidant activity of citrus peels

Orange, lemon and clementine peel extracts obtained under optimum extraction conditions deduced by DOE and RSM approaches were characterized in terms of total polyphenol, total flavonoid and

antioxidant capacity, following the experimental procedures described in Sections 2.5-2.8. The obtained results were compared statistically by using one way-ANOVA.

According to data included in Table 3, no significant differences were observed between the TPC and TFC values estimated respectively for orange, lemon and clementine peels, thus indicating similar total contents in all studied extracts.

When TAA and EC_{50} values obtained from DPPH assays were analyzed by the ANOVA test, significant differences (p-values < 0.05) among orange, lemon and clementine peels were found. In order to analyze the difference pattern among means, the least significant difference (LSD) multiple comparison test was applied. It could be concluded that extracts from orange peels had significantly lower total antioxidant activity (estimated by the Mo reduction assay) than lemon and clementine ones, clementine presenting the highest TAA values.

Regarding the antioxidant capacities estimated by the DPPH assays (Table 3), the results found were slightly different when expressed as mg of extract per gram of dried peel (mg extract·g^{-1} DW) and mg of trolox equivalents per gram of dried peel (mg·TE·g^{-1} DW). In fact, estimated EC_{50} values (mg extract·g^{-1} DW) were significantly different at the 95% confidence level for all citrus peels, clementine extracts showing the highest radical scavenging ability, followed by the orange ones. However, no significant differences were observed between the trolox equivalent antioxidant capacity (TEAC) indexes calculated for orange and lemon peel extracts, which were significantly lower than those estimated for clementine. Therefore, the results obtained from DPPH assays again evidence the high antioxidant power of clementine compared to both orange and lemon peel extracts, which is an important parameter for medicinal bioactive components.

Although spectrophotometric methods have been widely used for estimation of total polyphenol and flavonoid contents, it is worth to mention that these assays are based on the recognition of various structural groups present in polyphenolic compounds, attending to their properties and reactivity (Nazck & Shahidi, 2004). Consequently, the interferences caused by substances such as proteins, carbohydrates, nucleic acids and amino acids, which can absorb and react likewise, reduce their

selectivity (Escarpa & González, 2001). In addition, their reproducibility is influenced by pH conditions and solvent, being an important source of variability (Nazck & Shahidi, 2004). DPPH free radical assay has also the limitation caused by colour interference and sample solubility (Oboh & Ademosun, 2012).

For all these reasons, spectrophotometric methods are useful only for an approximate estimation of total polyphenol and total flavonoid contents, as well as antioxidant capacity, being necessary to employ to more selective and robust methods, such as the chromatographic ones for obtaining the individual polyphenolic profile (Ignat et al., 2011).

3.4. Principal component analysis (PCA)

In order to summarize and to easily visualize all extraction results, data were subjected to PCA. This chemometric tool reduces the dimensionality of the multivariate data to two or three principal components (PCs), that can be visualized graphically, with minimal loss of information. For this purpose, the data matrix was decomposed into matrices of scores (coordinates of the samples) and loadings (polyphenols), providing information on samples and variables, respectively.

In the exploratory study, only including the extraction results derived from DOE, two principal components explained 84.2% of the cumulative variance, while 3 PCs explained a percentage of 95.9%. Relationships between samples and variables were investigated from the simultaneous study of scores and loadings, from the called bi-plot (Fig. 3a). This 3D graph revealed patterns and differences among citrus peel extracts; scores showed sample distributions and loadings explained the behavior of the variables and their correlations. As it can be seen, the loading directions for hesperidin and rutin are the same, but different to the loading directions of both *p*-coumaric and *trans*-ferulic acids. These loadings suggest that the extraction behavior of *p*-coumaric is similar to *trans*-ferulic, and on the same way are hesperidin and rutin ones. On the other hand, majority extracts are grouped and placed at the opposite direction of loadings (studied polyphenols) and thus these experiments are not characterized by high polyphenol contents. The highest extracted content

of hesperidin and rutin are achieved from orange peel (experiment OR18, Table S2), meanwhile the largest *p*-coumaric and *trans*-ferulic acids contents are obtained from clementine peel (experiment CL1, Table S4). In both cases temperature is fixed at 90 ºC but orange peel extraction requires lower time (10 min), and clementine peel extraction lower ethanol ratio (20 %, v). Experiments namely LE7 (Table S3) and CL2 (Table S4) also appear grouped in the 3D bi-plot, indicating that the extracted content from lemon and clementine is similar under specific polyphenol extraction conditions. In addition, the score of OR17 (Table S2) fall far to *p*-coumaric and *trans*-ferulic, meaning very low extracted contents. Finally, among all extraction conditions, CL7 (Table S4) provided the highest amount of hesperidin.

Therefore, each studied polyphenol could be obtained for specific peel and extraction conditions. In general, orange peel could be the most appropriate matrix to achieve bioactive hesperidin and rutin, while clementine the most adequate to recover both *p*-coumaric and *trans*-ferulic acids. Obviously, lemon peel is the least indicated as a source of valuable polyphenols although, according to Table 3, it presented similar TFC and TFC values to orange and clementine peels. This fact again evidences the useful of chromatographic methods versus spectrophotometric ones for sample characterization and maximum selectivity.

On the other hand, PCA was also applied to detect patterns between the amount of each polyphenol and the DPPH, TFC, TPC and TAA estimated values under optimum conditions for extraction. Figure 3b shows the 2D bi-plot obtained for objects (different peels) and the variables studied. The proximity of clementine peel extract to the loading vectors corresponding to *p*-coumaric and *trans*-ferulic acids revealed that clementine citrus peel was the residual waste with the highest amounts of these polyphenols, as can also be seen in Table 2. The figure also confirms important correlations between DPPH antioxidant capacity and hesperidin amounts, clementine again being the citrus peel sample showing the highest EC_{50} index (Table 3) and hesperidin amount (Table 2). In addition, important information could also be deduced considering the opposite direction of the vectors (*i.e.* rutin and TPC, or both *p*-coumaric and ferulic acids and TFC). As shown in Table 2 and Table 3,

clementine peels presented the highest amounts of *p*-coumaric and ferulic acids, but the lowest values of TFC, while orange peel extracts showed the highest rutin content but the lowest TPC and TAA estimated values.

3.5. Analysis of citrus peel extracts by LC–MS/MS

Complementary analyses by LC–MS/MS were performed to confirm unequivocally the identity of polyphenols extracted from evaluated citrus peels. The extracts obtained under the optimum extraction conditions, deduced from RSM and MRA (see Section 2.4), were subjected to HPLC coupled to electrospray ionization triple quadrupole MS. Positive and negative ion modes were accomplished in ESI-MS analysis. The negative ion mode was finally selected due to clear parent and fragment ion signal, and low background noise. The followed procedure in the negative ion mode was accorded to Section 2.10.

Once again gallic acid, myricetin and resveratrol were not detected at the method detection limits. Both *p*-coumaric and *trans*-ferulic acids were also detected by LC–MS/MS in all citrus peel extracts, but at concentrations below quantitation limits. This fact could be attributed to a low sensitivity frequently associated to the negative ionization mode (Zuo et al. 2017).

As a novelty, narangin was identified in the three studied matrices due to the high selectivity of the MS/MS analysis, allowing its quantification at levels of 1.72 $mg \cdot g^{-1}$ (clementine), 4.32 $mg \cdot g^{-1}$ (orange) and 115 $mg \cdot g^{-1}$ (lemon). The determined concentration of narangin for lemon was considerably higher than those quantities reported by other authors such as García-Castello et al. (2015), regarding narangin extraction from *Citrus paradisi L*. residues by CSE (24 $mg \cdot g^{-1}$) and UAE (36 $mg \cdot g^{-1}$).

Otherwise, quercetin was only identified in the orange peel extracts when MS/MS was used, attending to the higher sensibility of this detection technique for this particular analyte. However, quercetin was observed at very low concentrations (< LOD). Conversely, great amount of hesperidin extracted from lemon, orange and clementine peels was confirmed at similar

concentration ratios to the ones derived from cLC-DAD analysis. In addition, according to the results obtained by cLC-DAD, rutin was confirmed in all studied matrices, but at concentration levels below LC-MS/MS quantitation limits.

Therefore, as derived from the MS/MS analysis, evaluated peels could be considered a scarce source of rutin and/or quercetin, the last one only in orange peel case, but surprisingly they could be a rich source of naringin, apart from hesperidin, which has been extracted at quite elevated amounts, especially from lemon peels.

4. Conclusions

A fast, sustainable and probably economic method with low instrumental requirements and operation simplicity has been proposed for the extraction of value-added polyphenols from different citrus peels. The proposed extraction procedure could be easier to perform than other extraction techniques such as UAE, MAE or ASE, especially at an industrial scale, maybe also involving lower economic costs. For obtaining the maximum extraction efficiency for all studied analytes, temperature should be fixed at 90 ºC; although, for a specific polyphenol, temperature could be decreased down to 62 ºC and the ethanol ratio reduced to 20% (v) in some extraction conditions, and thus decreasing the cost associated with heating or organic-solvent using. However, the global cost of the extraction process at industrial scale due to the heating or solvents should be compensated by the economic benefit obtained from the polyphenols extracted. In an approximate estimation, 100 g of synthetic *trans*-ferulic acid could have a cost of 116 €, 100 g of hesperidin 124 €, 100 g of rutin 128 €, and 100 g of *p*-coumaric acid 410 €. Using the proposed methodology, each gram of clementine peels allows obtaining up to 673 mg of hesperidin, and 4.7 mg of rutin each gram of orange peels. These extracted quantities are considerably higher those that reported by other authors.

In general, the obtained results suggested that clementine peel could be a rich and abundant source of health beneficial bioactive phenolic compounds, and especially hesperidin, while orange peel

could provide higher contents of rutin. Furthermore, the LC-MS/MS analysis allowed us to confirm especially high amounts of naringin, extracted mainly from lemon peels. In all cases, hesperidin, which has demonstrated interesting therapeutic functions as protector against UVA irradiation and oxidative stress, was the most abundant extracted polyphenol from all citrus matrices.

On the other hand, experimental design and complementary chemometric tools (RSM, MRA, multifactorial-ANOVA and PCA) have demonstrated to be greatly effective to analyze and to summarize great amount of data, as well as to facilitate the recognition of relevant underlying information and to find out the best extraction conditions. Thus, optimum conditions derived from this study could be used as a guideline information for further pilot plant-scale trials and industrialization of the extraction process. In addition, both cLC-DAD and LC-MS/MS proposed methods, have provided a simple and efficient strategy for rapid characterization and quantification of recovered phenolic natural products.

Therefore, evaluated citrus peel waste could be reused and valorized helping to minimize the environmental impact and be converted into value-added products, with potential interest for the development of functional foods, cosmetics or likely preventive therapies for some diseases, adding a value to the citrus processing waste and companies.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Captions

Fig. 1. Estimated normalized response surfaces obtained for the content (μg·g^{-1}) of *trans*-ferulic acid extracted from lemon peels (a) and *p*-coumaric acid extracted from orange peels (b), both at a temperature of 90 ºC (model factor C), different extraction times (model factor A) and different EtOH-H_2O proportions of extraction solvent (model factor B). The figure also includes the estimated response surface for the desirability function provided by the MRA analysis for clementine peels (c).

Fig. 2. Multi-factorial ANOVA plots showing the influence of extraction parameters and type of citrus peel on individual polyphenol content. *p*-Values (P) lower than 0.05 indicated a statistically significant effect at a confidence level of 95 %. OR (orange), LE (lemon), CL (clementine).

Fig. 3. **a)** Biplot of the simultaneous evaluation of the relationship between scores (extraction experiments from orange, lemon and clementine peels) and loadings (polyphenols). OR (orange), LE (lemon), CL (clementine). Numbers in the figure indicate the conditions of the extraction experiments according to Tables S2-S4. **b)** Biplot of the simultaneous evaluation of the relationship of scores (orange, lemon and clementine peels) and loadings (polyphenols extracted and total parameters estimated under optimum extraction conditions). TPC (total polyphenol content), TFC (total flavonoid content), TAA (total antioxidant activity), DPPH (scavenging activity of extracts against 1,1-diphenyl–2 picrylhydrazyl free radical, expressed as EC_{50} mg extract·g^{-1} sample DW).

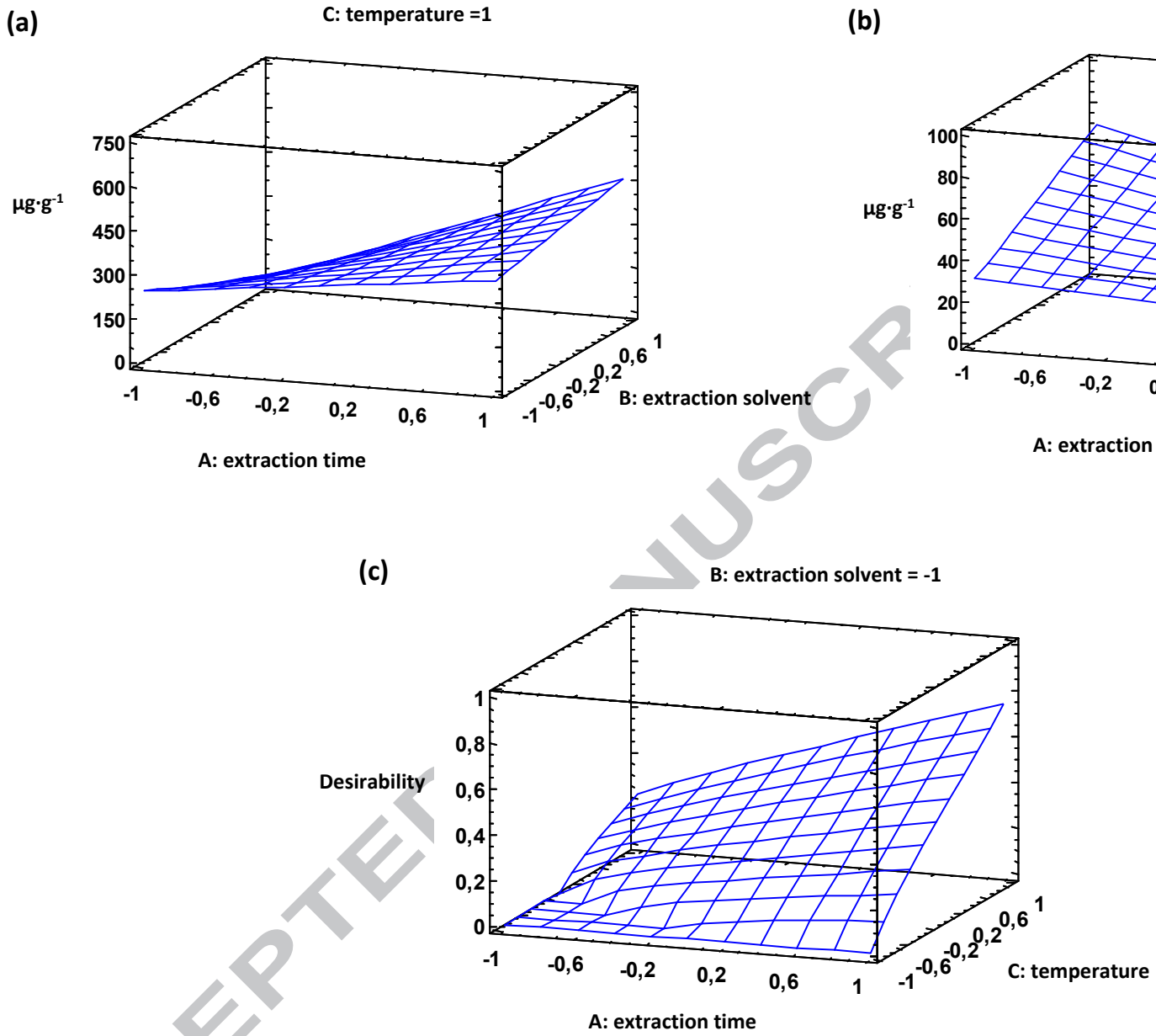


Fig. 1

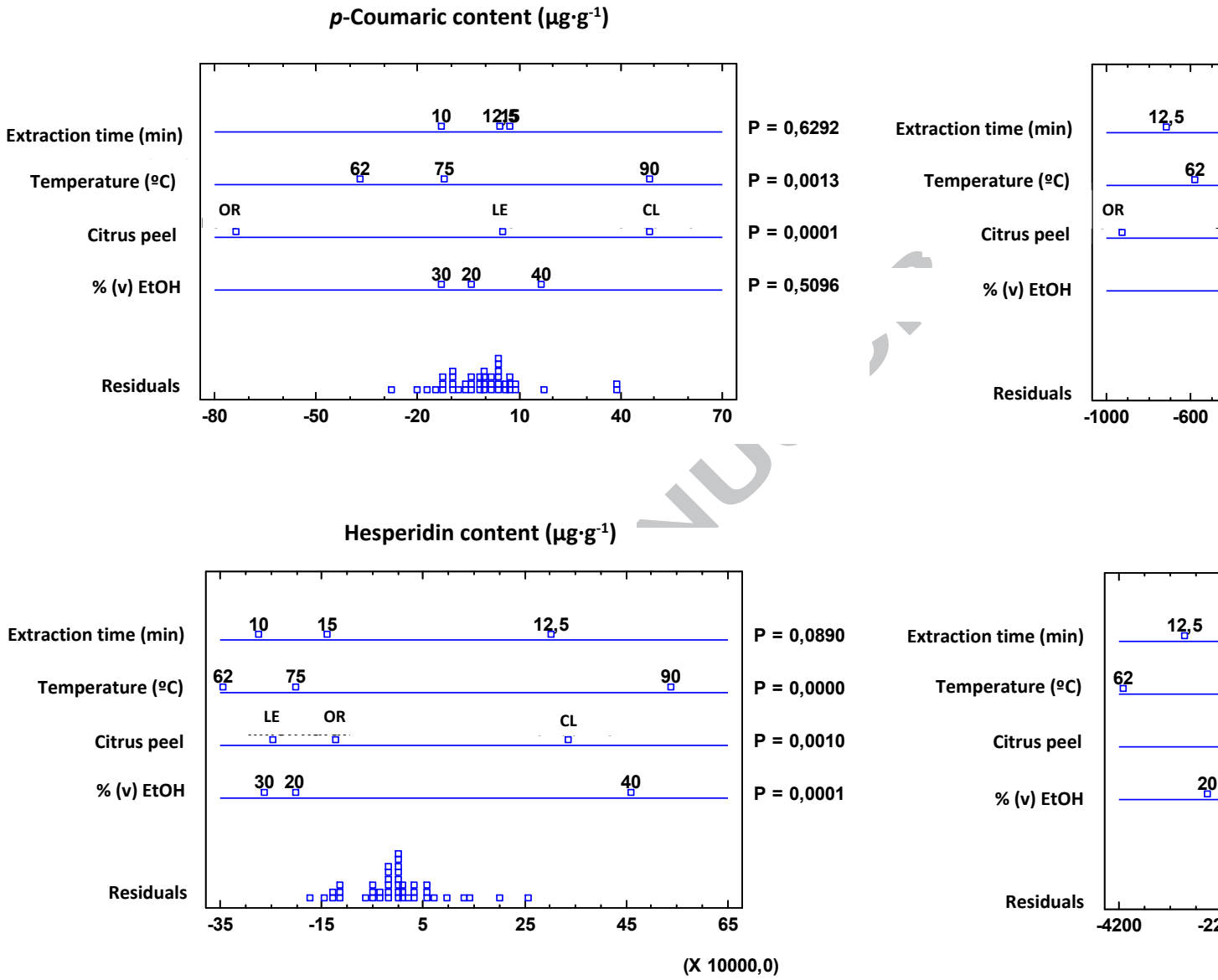
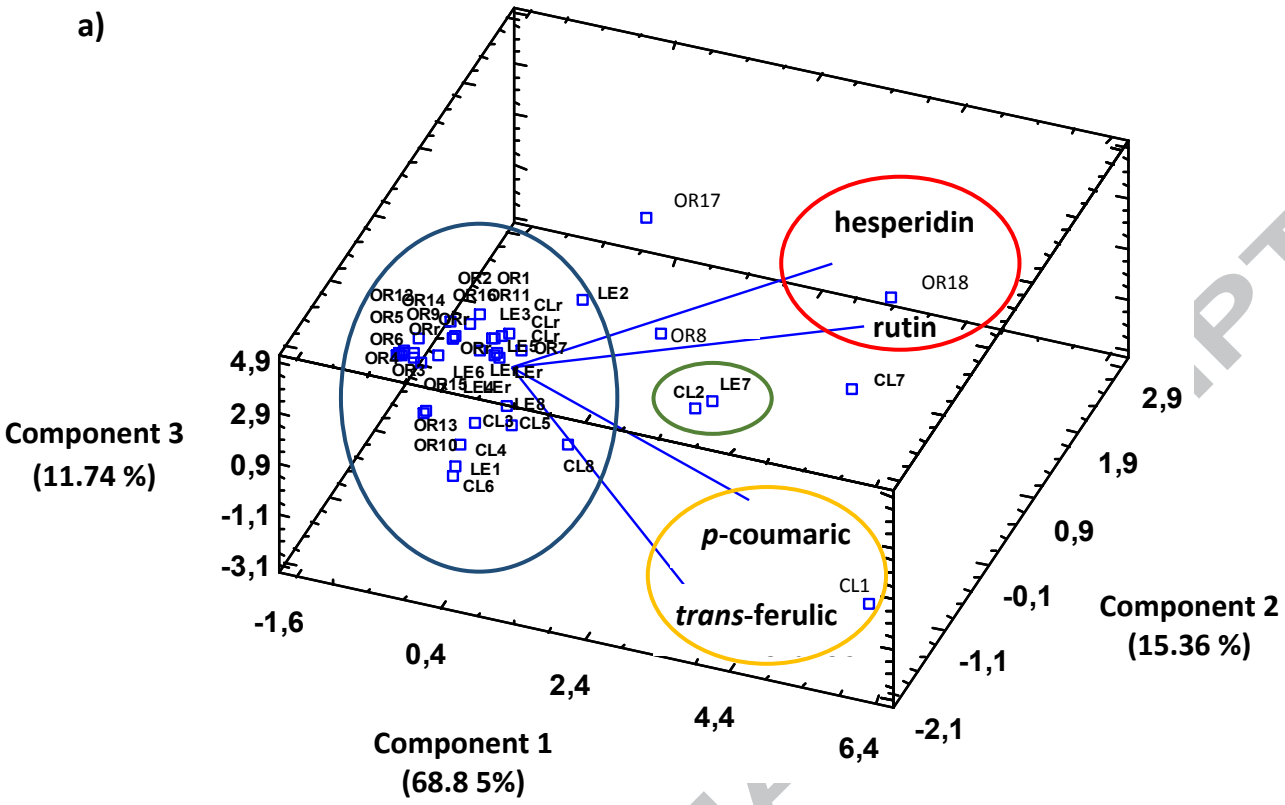


Fig. 2



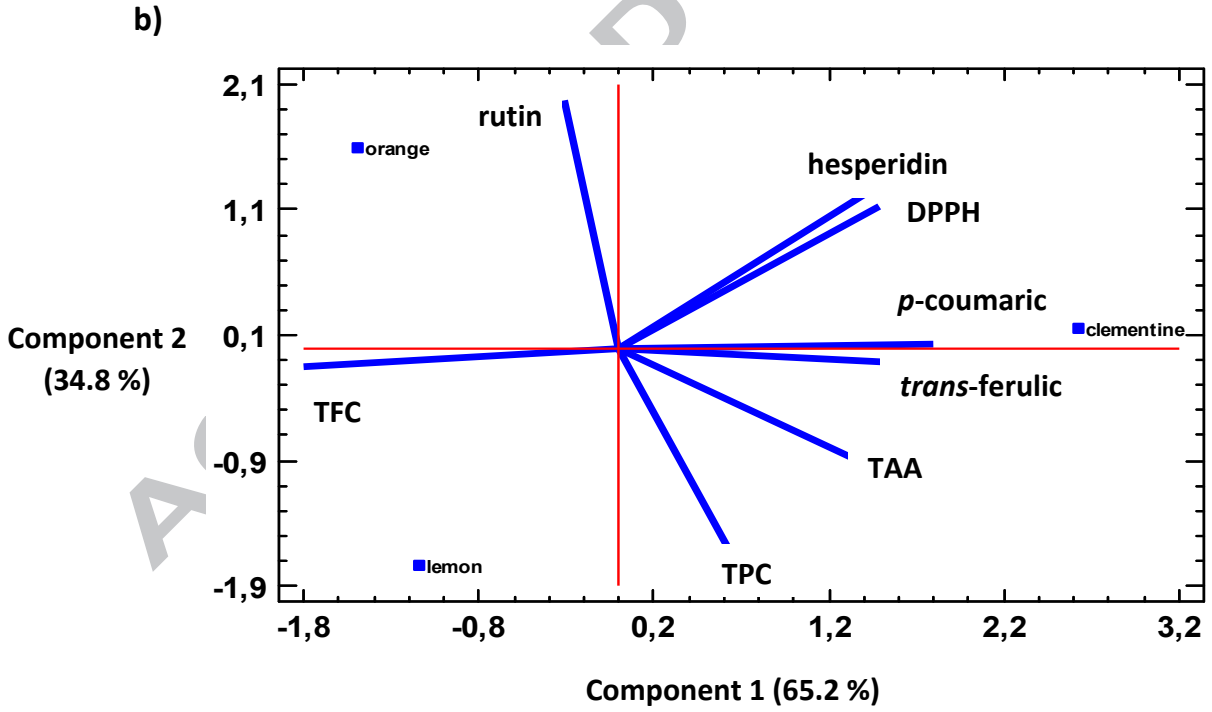


Fig. 3

Table 1. Analytical parameters of the cLC-DAD employed for determination of bioactive polyphenols.

	LOD ($\mu g \cdot L^{-1}$)	Linear range ($\mu g \cdot L^{-1}$)	Calibration equation $y = ax + b$			Intra-day repeatability (n=3) RSD (%)		Inter-day repe (three days) R
			a	*b*	R^2	*k* [a]	Area [a]	*k* [a]
	4.6	18-100	3.359	1.2037	0.9932	0.7; 0.6	3.3; 9.8	1.5; 2.3
id	1.2	5-100	54.535	7.9930	0.9926	0.9; 1.5	2.7; 1.7	4.7; 2.9
cid	2.8	10-100	-9.185	3.1360	0.9956	0.2; 1.3	3.4; 2.2	4.1; 2.6
	3.1	10-100	16.312	1.3246	0.9942	0.9; 1.4	2.0; 1.5	6.1; 2.9
	30	100-500	9.0085	0.1161	0.9918	1.7; 1.0	0.7; 7.0	1.7; 3.1
	3.2	10-100	14.546	1.0419	0.9925	1.0; 1.5	2.0; 3.8	1.0; 3.0
	22	75-500	-12.510	0.7235	0.9927	1.4; 0.9	0.3; 0.3	7.4; 3.0
	3.1	10-100	30.968	6.2906	0.9930	1.8; 1.0	4.7; 4.5	6.2; 3.0

[a] Values obtained at low and high concentration levels, respectively.

Table 2. Extraction results obtained from experimental design and RSA for orange, lemon and clementine peels. Goodness of fit, significant identified factors and sign effects (in parenthesis), and maximum predicted values are indicated.

p-Coumaric acid			*trans*-Ferulic acid			Rutin			Hesperidin
Orange	Lemon	Clementine	Orange	Lemon	Clementine	Orange	Lemon	Clementine	Orange
0.849	0.984	0.783	0.721	0.802	0.704	0.689	0.966	0.833	0.841
AB (-) A (-) C (+) B (+) BC (+)	C (+) AC (+) A (+)	C (+)	-	C (+)	-	C (+)	B (+) C (+) BC (+)	C (+)	C (+) B (+) BC (+)
:									
10 40:60 90	15 40:60 90	15 20:80 90	10 40:60 90	15 40:60 90	15 20:80 90	10 40:60 90	15 40:60 90	15 20:80 90	10 40:60 90
0.070	0.072	0.100	0.293	0.462	1.38	4.70	3.30	3.88	498

[a] Model factors: A = Extraction time, B = Ethanol-water proportion of extraction solvent, C = Temperature. Factors placed on the top of the column are the experimental factors with higher statistical weight in the model equation. DW: dry weight.

Table 3. Comparison of the TPC, TFC and antioxidant activity (using reduction of Mo assay and DPPH radical scavenging values) of citrus peel extracts. Data are expressed as mean ± standard deviation (n = 3).

TPC (mg GAE·g^{-1} DW)	TFC (mg QE·g^{-1} DW)	TAA (mg GAE·g^{-1} DW)	DPPH assay, EC_{50} index (mg extract·g^{-1} sample DW)	TEAC (mg TE·
3.9 ± 0.2 [a]	17.6 ± 0.6 [a]	3.7 ± 0.7 [a]	22 ± 3 [a]	1.4 ± 0.1 [a]
5.9 ± 0.4 [a]	18 ± 2 [a]	17.9 ± 2.0 [b]	13 ± 3 [b]	1.1 ± 0.2 [a]
5.5 ± 1.7 [a]	16.5 ± 1.2 [a]	28.2 ± 1.9 [c]	31 ± 2 [c]	2.0 ± 0.2 [b]

Mean values with different letters in the same column indicate significant differences at *p*-values < 0.05, according to ANOVA and Fisher LSD test.

TPC (total polyphenol content), GAE (gallic acid equivalents), TFC (total flavonoid content), QE (quercetin equivalents), TAA (total antioxidant activity), DW (dry weight), EC_{50}: concentration needed to reduce 50% of DPPH•, TEAC (trolox equivalent antioxidant capacity), TE (trolox equivalents).

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Polyphenols were effectively extracted from citrus peels in short extraction times
- High amounts of hesperidin were obtained especially from clementine peels
- Extraction conditions were deduced by DOE on the basis of individual polyphenols
- PCA was a useful strategy for analysis and evaluation of extraction data
- Identity of extracted polyphenols was corroborated by LC-MS/MS