Green-sustainable extraction techniques for the recovery of antioxidant compounds from "citrus Limon" by-products

Green-sustainable extraction techniques for the recovery of antioxidant compounds from "Citrus limon" by-products

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Abstract

In this work, optimized techniques of conventional, ultrasound and microwave-assisted extraction were applied for the recovery of antioxidant compounds from lemon by-products (*Citrus limon L*). Specifically, the effect of solvent, temperature, microwave power, time and their interaction on the extraction was investigated. Among the tested solvents, the hydroalcoholic mixture (ethanol:water, 50:50) was the optimal one for all extraction techniques: in particular assisted by ultrasounds at 70°C for 30 minutes (total phenolic content: 6.93 mg GAE g^{-1}, total flavonoids: 2.07 mg CE g^{-1}, ABTS assay: 18.36 µM TE g^{-1}). Also, the other techniques allowed to obtain valuable extracts, although with relative lower amounts. The analyses of individual phenols revealed hesperidin and eriocitrin as the main compounds (respectively about 1650 and 1150 mg kg^{-1}) after ultrasound assisted and conventional extraction. Results of this work can be useful to valorise an industrial by-product by sustainable techniques for the high-added value substances recovery.

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Introduction

Food industry produces considerable quantity of solid and liquid waste, obtained from the conversion of feedstock into final products, representing a severe environmental issue due to their content of organic substances. On the other hand, interesting perspectives arise from the huge amount of food by-products. Among fruit and vegetables, citruses are one of the world's most abundant fruit crop and the processing of fruits [1], such as lemon, produces considerable quantities of by-products, often including useful compounds in their peels, pulps, and seeds [2], which could be extracted and utilized as natural antioxidants to avoid oxidation of some foodstuff or may be applied in functional foods formulations [3,4]. Among polyphenols, flavonoids are characterized by relevant biological actions, which include antioxidant, anti-inflammatory, anticancer, antiviral, and anti-mutagenic activities [5].

Extraction represents the first step to get valuable compounds from a food matrix and several optimized techniques can be developed to obtain them from peel wastes, by conventional solvents [6], microwave-assisted extraction [7,8], assistance with cyclodextrin [9], enzymes [10], ultrasounds [11], and subcritical water [12]. Generally, conventional techniques (solid-liquid extractions) have been broadly used to get bioactive compounds from natural matrices, particularly the maceration extraction. This type of extractions can imply many drawbacks such as: use of high temperatures and long extraction times; low selectivity and low extraction yield; high energy input, safety hazards and environmental risks. The application of alternative techniques is useful to reduce the disadvantages and increase the extraction yield [13,14]. On the other hand, ultrasound -assisted extraction represents a promising technique to reduce the extraction time and increase the extraction

yield and quality, as well as microwave-assisted extraction, which could be considered an alternative extraction technique that combines microwave and traditional solvent extraction.

In this context, organic solvents, as ethanol, methanol and their mixture with water are generally used for the extraction of antioxidant compounds from citrus pulp and peel. Although lots of organic solvents, like methanol, are efficient solvents for extraction of polyphenols, ethanol categorized under GRAS (Generally Recognized as Safe) is preferred because of its application in the food system. Water and hydroalcoholic mixture are the most employed solvents in food grade extraction: presence of water in solvent might lead to an increase in the extraction rate since water could be helpful to improve distension of plant material, which allows an increase in the contact surface area between food matrix and solvent [15]. Indeed, the yield of antioxidant compounds from plants is correlated to the polarity, solubility, as well as specific extraction parameters such as solvent nature and concentration, temperature, and time [16].

Furthermore, in citrus peels, phenolic acids are often related to several plant components through ester and glucoside bonds, while flavonoids can be either in the free (aglycones) or bound (glycoside) forms, with the former having higher antioxidant properties compared to the latter. For this reason, applying heat on citrus by-products during the extraction process may boost the liberation of polyphenols by breaking down both ester and glucoside bonds, which tend to be very stable at room temperature [17]. The extraction rate depends not only on the nature of the applied solvents and the solvent: sample ratio, but also on the extraction time and temperature and the chemical composition and physical characteristics of the matrices [18, 19]. As reported by De Bruno et al. [20], a longer extraction time does not always correspond to a higher extraction yield.

Therefore, one of the main purposes of this investigation was focused on the effect of the working conditions (solvent, time, and temperature) on the extraction of bioactive compounds from lemon by-products in order to obtain extracts with a high quantity of antioxidant compounds that, consequently, express an equally high antioxidant activity. Moreover, this study aimed to develop

environmentally friendly and food grade techniques (conventional, ultrasound and microwave) with higher extraction efficiency.

Materials and Methods

Materials

Lemon by-products samples (*Citrus limon (L.) Osbeck*) called also "pastazzo and/or Lemon pomace" were picked during 2018/2019 crop season, at the Agrumaria Reggina company located in Reggio Calabria (Italy). By-products from the extraction of lemon juice and essential oils consisted of lemon peel, pulp and seeds. Afterwards, they were transported to the Food Technology laboratory of the Mediterranea University of Reggio Calabria where they were immediately dried at a temperature of 50°C up to a final moisture content of 12% and stored in polyethylene bags under vacuum to avoid rehydration until subsequent extraction procedures of the bioactive compounds.

Chemicals

Standards of gallic acid, *p*-coumaric acid, ferulic acid, eriocitrin, narirutin, hesperidin, neohesperidin, naringin were purchased from Merck (Darmstadt, Germany). Apigenin, caffeic acid and rutin were purchased from Extrasynthèse (France). The solvents used for chromatographic analysis (methanol, water, and acetonitrile) were ultra-high-performance liquid chromatography (UHPLC)-MS grade (Carlo Erba, Italy). 2,2′- Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2- diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu's phenol reagent, and Trolox were purchased from Sigma Chemical Co. (USA).

Experimental procedure

The recovery of bioactive compounds has been carried out through three different extraction techniques: conventional solid-liquid extraction, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). A simplified outline of the experimental procedures was represented in Figure 1.

The extractions were performed using two different food grade extraction solvents, water (H_2O) and ethanol: water mixture (EtOH: H_2O, 50:50). Three temperatures (25, 40 and 70°C) were tested. The extraction times for MAE were 5 and 15 minutes, and those for conventional and UAE were: 30, 60 and 120 minutes.

Conventional Solid-Liquid Extraction

The extraction was performed according to Papoutsis et al. [21], with some modifications. Briefly, 10 g of dried and ground lemon by-products and 50 mL of solvent (1:5, w:v, ratio) were mixed and placed on a heating magnetic stirrer for the desired times and temperatures, monitored by a digital thermometer. Subsequently, samples were centrifuged (NF 1200R, Nüve, Ankara, Turkey) at 5000 rpm for 5 min at 4°C, filtered through a Büchner apparatus with 0.45 µm filter paper and the resulting extracts were made up to volume of 50 mL with the respective extraction solvent. The extracts were then filtered with 0.45 µm nylon filters and stored at -20°C until subsequent analyses.

Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) was performed using a Sonoplus Ultrasonic homogenisers, Series 2000.2, HD 2200.2 (BANDELIN, Ultraschall seit 1955), composed of an inox jug with a

capacity from 20 to 900 mL, a VS 70 T probe of 13 mm and a maximum permitted amplitude setting of 100%. The ultrasonic generator converts the mains energy input (mains frequency 50 or 60 Hz) into high-frequency energy (frequency of 20 kHz), guaranteeing the reproducibility of the process parameters and the validation of the extraction procedure.

For the extraction, 20 g of sample and 100 mL of solvent were mixed (1:5, w:v, ratio) and the desired temperature conditions have been achieved through the control and regulation of radiation rate over time, in terms of radiation amplitude (expressed as percentage) and radiation rate per second. The temperature trend was monitored throughout the extraction time with a digital thermometer. It was found that by applying a specific radiation amplitude (ω) and pulsation time, in relation to the frequency of 20 kHz ± 500 Hz, it was possible to obtain a corresponding temperature for the entire extraction process:

- 30 minutes at 25°C (ω = 10%, pulsation time on 1s off 15s), 40°C (ω = 20%, pulsation time on 1s off 5s), 70°C (ω = 50%, pulsation time on 1s off 1s);
- 60 minutes at 25°C (ω = 10%, pulsation time on 1s off 15s), 40°C (ω = 25%, pulsation time on 1s off 5s), 70°C (ω = 50%, pulsation time on 1s off 1s);
- 120 minutes at 25°C (ω = 10%, pulsation time on 1s off 30s), 40°C (ω = 20%, pulsation time on 1s off 10s), 70°C (ω = 50%, pulsation time on 1s off 1s).

Subsequently, each sample was treated as described above for conventional extraction.

Microwave -Assisted Extraction

The extraction was performed according to Li et al. [7], with some modifications. In the Microwave Digestion System (ETHOS EASY, Millestone, Bergamo, Italy) used for the extraction, the thermal conditions have been achieved through control (by easyTEMP thermal sensor - ATC-CE) and regulation of the applied microwave power (Watt), acquiring for each applied power the corresponding temperature. Specifically, it was found that:

- 250 W corresponds to 25°C;
- 500 W corresponds to 40°C;
- 800 W corresponds to 70°C.

In brief, 2.5 g of dried lemon by-products were ground and dissolved in 25 mL of solvent extraction (1:10, w:v, ratio), homogenized with ultra-turrax apparatus (IKA T 25, Staufen, Germany) and transferred into PTFE-TFM vessels of 100 mL (SK-15 easyTEMP, high-pressure rotor). The vessels were placed at the centre of the microwave apparatus, heated to the selected temperature in 3 minutes and, then, held at temperature for a certain extraction time (5 or 15 minutes), according to the experimental design.

After microwave heating, the mixtures in the extraction vessels have been left to cool down to room temperature in 10 minutes. Subsequently, each sample was treated as described above for the other extraction techniques.

Analytical Methods

Total Phenolic Content (TPC)

TPC was determined according to the method reported by González-Molina et al. [22], with appropriate modifications. 0.2 mL of extract were placed inside a 25 mL flask and mixed with 5 mL of deionized water and 1 mL of Folin-Ciocalteu reagent. After 8 minutes, 10 mL of saturated sodium carbonate solution (Na_2CO_3) at 20% (w/v) were added and made up to volume with deionized water. At the same time, the solution used as a blank was prepared, without the addition of the sample. The mixtures were incubated for two hours at room temperature and in the dark. The absorbance of the samples was measured at 765 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.)

and comparing with a gallic acid calibration curve (concentration between 1 and 10 mg L^{-1}). The results were expressed as mg of gallic acid g^{-1} (mg GAE g^{-1} d.w.) of lemon by-products dry weight. Total polyphenol yield was calculated with the following eq.

$$TPC\ Y_{(mg\ GAE\ g\text{-}1\ dw)} = \frac{(TPC \times V)}{m}$$

.

Where: V is the extraction volume and m is the dry weight of lemon by-products (g).

Total Flavonoid Content (TF)

The total flavonoid content (TF) was quantified on the obtained extracts by the method described by Papoutsis et al. [21], with some modifications.

In brief, 0.2 mL of extract, 1 mL of deionised water and 0.15 mL of 5% (w/v) $NaNO_2$ were mixed in a 5 mL flask and incubated at room temperature for 6 minutes. Subsequently, 0.15 mL of 10% (w/v) $AlCl_3$ were added and incubated at room temperature for 6 minutes. Then, 2 mL of 4% NaOH (w/v) and 0.7 mL of deionised water were added and finally the mixture was made up to volume with deionised water. At the same time, a solution used as a blank was prepared with the same amounts of reagents but without the addition of the sample. The mixture was incubated in the dark for 15 min. The absorbance was measured at 510 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.) and comparing with a catechin calibration curve (concentration between 1 and 50 mg L^{-1}). The results were expressed as mg of catechin g^{-1} (mg CE g^{-1} d.w.) of lemon by-products dry weight. Total flavonoids yield was calculated with the following eq.

$$TF\ Y_{(mg\ CE\ g^{-1}\ dw)} = \frac{(TF \times V)}{m}$$

Where: V is the extraction volume and m is the dry weight of the lemon by-products (g).

Antioxidant Activity Determination

DPPH Assay

The DPPH assay was performed as reported by Brand-Williams et al. [23], which is based on the reaction between the DPPH· (2,2-diphenyl-1-picrylhydrazyl) and antioxidant compounds in the samples, resulting in discoloration of the reaction solution due to the disappearance of the radical. In a cuvette, 50 µL of the extract (aqueous and hydroalcoholic) properly diluted were added to 2950 µL of a 6 x 10^{-5} M of methanol solution of DPPH· and left in darkness for 30 min at room temperature. The absorbance was measured at 515 nm, against methanol as blank, using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.).

The results were expressed as µM Trolox equivalents g^{-1} of lemon by-products dry weight (µM TE g^{-1} d.w.), comparing with a Trolox calibration curve (from 3 to 18 µM).

ABTS Assay

The antioxidant activity of the extracts was determined by ABTS (2,2'-azino-bis acid (3-ethylbenzothiazolin-6-sulfonic acid) assay, a spectrophotometric discoloration method [24]. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM

potassium persulphate ($K_2S_2O_8$) solution and was incubated at room temperature for 12 hours in the dark to achieve a stable value of absorbance: the reaction between $ABTS^+$ and potassium persulphate determines the direct production of a blue-green chromogen. The resulting $ABTS^+$ solution was diluted with ethanol and showed an absorbance of 0.70 (±0.02) at 734 nm.

The reaction mixture was prepared by mixing 25 µL of aqueous extract and 2975 µL of $ABTS^+$ solution; the hydroalcoholic extracts required different reaction ratios, such as 10 µL of extract and 2990 µL of $ABTS^+$ solution. The absorbance was measured after 6 minutes in the dark at 734 nm using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.).

The quenching of initial absorbance was plotted against the Trolox concentration (from 3 to 18 µM) and the results were expressed as µM Trolox equivalents g^{-1} of lemon by-products dry weight (µM TE g^{-1} d.w.).

Identification and Quantification of Antioxidant Compounds

Identification and quantification of antioxidant compounds was performed in each extract following Romeo et al. [25], with some modifications. Chromatographic system consisted in UHPLC PLATINblue (Knauer, Berlin, Germany) provided with a binary pump system, Knauer blue orchid C18 column (1.8 µm, 100 x 2 mm) coupled with a PDA–1 (Photo Diode Array Detector) PLATINblue (Knauer, Berlin, Germany) and Clarity 6.2 software.

Extracts were filtered through a 0.22 µm nylon syringe filters (diameter 13 mm) and then 5 µL were injected in the system. The mobile phases used were (A) water acidified with acetic acid (pH 3.10) and (B) acetonitrile; the gradient elution program consisted in 0–3 min, 5% B; 3–15 min, 5%–40% B; 15–15.5 min, 40%–100% B. Ultimately, restoration of the initial conditions was reached during analysis maintaining the column at 30°C. For the quantification of each antioxidant compounds,

external standards (concentration between 1 and 100 mg kg^{-1}) were used, and the results were expressed as mg kg^{-1} of lemon by-products dry weight (mg kg^{-1} d.w.).

Antimicrobial Activity of Extracts

The three foodborne pathogens Listeria monocytogenes (ATCC 13932 strain), Escherichia coli (ATCC 8739 strain) and Salmonella enterica (wild strain) were used as test organisms to determine antimicrobial activity of the best selected extracts via the agar diffusion method based on the inhibition zones, at the "University of Messina", Veterinary Science Department. 10 µL of each extract were inoculated onto Petri plates on the surface of solid soft agar with the test organism (TSA, Tryptic Soy Agar, for L. monocytogenes; MH, Mueller Hinton Agar, for E. coli and S. enterica) and incubated at 37°C for 24 h. EtOH:H_2O 50% mixture was used as control. Tests were repeated in duplicate. The results were expressed as mm of the halo diameter resulting from the inhibition zone taken as a measure of the antimicrobial activity of the extract.

Statistical Analysis

All the experimental results were expressed as mean value (n=4) ± standard deviation. SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) was used for data statistical elaboration. Multivariate and One-way analysis of variance (MAVOVA and ANOVA) with Tukey's post hoc test at $p<0.05$. Pearson's correlation test was employed for the determination of correlation coefficients (r) among TPC, TF and antioxidant assays (DPPH and ABTS).

Results and Discussion

296 *Conventional Solid-Liquid Extraction*

297

298 Multivariate data analysis evidenced a significant difference ($p<0.01$) between the effect of water
299 and hydroalcoholic mixture when considered jointly on the dependent variables, whereas the tested
300 times affected only TF (Table 1). Consequently, a separate ANOVA was conducted for each
301 dependent variable, with each ANOVA evaluated at an alpha level of 0.05.
302 In this regard, a different TPC extraction yield between the two types of extraction solvents used
303 was highlighted. In particular, the hydroalcoholic one promoted a higher efficiency for TPC than
304 water (Table 2, Fig.2). These results are confirmed by De Bruno et al. [20], who reported that the
305 water/ethanol mixture increases phenolic recovery from agricultural by-products.
306 Using solvent W, after 30 and 60 extraction minutes a decrease of TPC was observed with
307 increasing extraction temperature in the aqueous extracts obtained. The reduction could be
308 explained by the possible degradation of phenolic compounds at any considered temperature as the
309 extraction time increases [26]. Temperature of 25° C was considered the most appropriate. After 120
310 minutes of extraction with solvent W, the recovery was the lowest without thermal differences. The
311 maximum recovery of total phenolics in water extracts (W) was 2.96±0.11 mg GAE g^{-1} d.w. (30
312 min, 25°C), with higher results than some data reported in literature [27].
313 Hydroalcoholic extraction showed different behaviour. The obtained extraction yield is in
314 accordance with literature data related to the phenolic extraction from lemon by-products [28]. Our
315 results showed that the recovery of phenolic compounds linearly increased with the increase of
316 extraction temperature at all the considered extraction times (Table 2). Despite the similar ($p>0.05$)
317 TPC yield after 120 minutes at both 40°C and 70°C, the multivariate statistical analysis evidenced
318 no time effect on TPC values regardless the extraction temperature, as well as on the showed
319 antioxidant activity. As reported by Barrales et al. [29], the polyphenols content in ethanol increases
320 as the temperature increases, probably because of the decrease in surface tension, which represents
321 a factor that regulates the penetration of the solvent into the solid matrix. Moreover, higher

temperature increases the diffusivity of phenolic compounds into the solvent boosting their transport, even if at 70°C we should approach the boiling point of the W/EtOH azeotropic (78.4°C), which composition is highly dependent on temperature and pressure.

About the total flavonoid content (TF), highly significant differences ($p<0.01$) were found among samples at both extraction solvents. It was evident in the hydroalcoholic one, with a maximum total amount of 2.22±0.29 mg CE g^{-1} d.w. in extract obtained at 70°C for 120 minutes. Such value was consistent with that found by Papoutsis et al. [21] by extraction on freeze dried lemon peel.

In this study, TPC and TF showed similar patterns of extractability, with significant variations in the interaction effects by the independent variables ($p<0.05$): as the temperature increases, the extraction rate in ethanol 50% increases significantly at the same time.

Regarding the antioxidant activity of the extracts, it was tested by two assays, based on $DPPH^{\cdot}$ and $ABTS^{+}$ extinction, which often do not give the same results because of the two different action mechanisms and the two distinct radicals involved. This is the reason why it could be appropriate to perform both antiradical tests [20].

The aqueous extracts (W) did not show a significant difference in the expression of antioxidant activity for both assays performed, even if the correlation between polyphenols and flavonoids compounds and the DPPH assay was high, $r=0.92$ and $r=0.83$, respectively. This is possible since a high phenolic content is not necessary characterized by a high antioxidant capacity, which also depends on the structure and interaction among the extracted phenolic compounds.

For the conventional solid-liquid extraction, the hydroalcoholic mixture and the highest temperature (70°C), were considered as the most appropriate parameters for the extraction of antioxidant compounds from dried lemon by-products, at any time among those considered, in terms of the highest TPC and TF values (Table 2).

Ultrasound - Assisted Extraction

The multivariate statistical analysis (Table 1) shows that in ultrasound-assisted extraction all individual independent variables and the interaction of them significantly affected ($p<0.05$) TPC and TF, as well as the expression of the antioxidant activity performed by ABTS and DPPH assays. The lack of significance of the DPPH assay suggests the absence of interactions among the independent variables in the studied range.

Consequently, a separate ANOVA was conducted for each dependent variable, with each ANOVA evaluated at an alpha level of 0.05.

Regarding the aqueous extracts (W), there was a highly significant difference ($p<0.05$) in the extraction yield of TPC and TF, which decreased with the extension of ultrasonic extraction time and temperature (Table 3). The application of high ultrasonic intensity may result in degradation effects. Indeed, the increment in amplitude of ultrasonic radiations had both positive and negative effects on the extraction yield. More precisely, the highest TPC and TF values (5.91±0.20 mg GAE g^{-1} d.w. and 2.30±0.08 mg CE g^{-1} d.w., respectively), were obtained with temperature of 25°C and extraction time of 60 minutes. As described by Papoutsis et al. [21], extraction temperature could have a significant negative effect on TPC yields, suggesting that an extraction temperature higher than the optimum leads to a decrease in TPC. The degradation of cell walls may expand as the temperature increases, follow up on release of both phenolic compounds and enzymes involved in polyphenols oxidation (i.e., peroxidase and polyphenol oxidase). Indeed, our results refer that the extraction yield was decreased by a further increment of the process intensity level, up to 70°C for 120 minutes, leading to the lowest TPC and TF values, respectively of 1.31±0.04 mg GAE g^{-1} d.w. and 0.25±0.05 mg CE g^{-1} d.w. of lemon by-products.

In addition, this aspect could be attributed to the simultaneous effects of the high radiation amplitude and the extended extraction time, producing several temporary hot spots through the collapse of the cavitation bubbles and an increase in temperature and pressure, which leads to the destruction of polyphenols in the UAE process [30, 31].

On the antioxidant activity showed by the W extracts, there was a high positive linear correlation between polyphenols (r=0.84) and flavonoids (r=0.88) compounds and the DPPH assay. Otherwise, a lower correlation between TPC and ABTS assay (r=0.67) was detected.

Contrary to what previously described, in W/EtOH extracts the bioactive compounds extraction yield increased with increasing of temperature and time. Looking at Table 3, there was a clear temperature effect on extractions for 30 minutes, whose increase led to an increment in TPC. Equally, it was noted that the increase of the extraction time beyond 30 minutes at the same temperature led to an increase in the extraction yield of total polyphenols. Extraction temperature and time had a significant positive linear effect on the extraction process ($p<0.05$), implying that higher yields of TPC can be achieved by increasing ultrasonic radiation amplitude and/or extraction time in agreement with Khan et al. [32]. In fact, UAE is an acoustic cavitation extraction technique that combines ultrasound and traditional solvent extraction: sound waves induce intense shear forces that can break the cell walls and allow the solvent to penetrate the plant cells, intensifying the release of constituents [33; 11]. In comparison to water, ethanol is characterized by a higher heating efficiency when applied in an aqueous mixture and it is preferred thanks to its better capacity in solving the phenolic compounds [34].

Additionally, high positive correlations were found ($r>0.80$) between TPC and TF content and both antiradical assays. Indeed, high antioxidant capacity value corresponds to a high phenolic content, with a maximum of 21.28±0.29 µM TE g^{-1} d.w. and 8.25±0.24 µM TE g^{-1} d.w. of lemon by-products in W/EtOH extracts, for ABTS and DPPH assays, respectively.

The greater efficiency of UAE than conventional extraction agrees with results obtained by several authors in the extraction of polyphenols from different matrices [15, 35].

Microwave-Assisted Extraction

From multivariate data analysis, among the independent variables taken into consideration in this research, the extraction solvent and power showed a highly significant influence on the extraction process. Indeed, the multivariate statistical analysis shows (Table 1) that a different solvent (W or W/EtOH) and the combination of this variable with the extraction power significantly affected ($p<0.05$) the extraction yield of TPC and TF and the expression of the antioxidant activity (ABTS and DPPH assays). Extraction power also had a significant influence on the dependent variables considered in this study, with the only exception of the total flavonoid content. Contrary to solvent and power variables, the multivariate statistical analysis suggests a lack of significant influence by time, especially on TPC and ABTS variables, and by the interaction of solvent and extraction time on the extraction process in the studied range.

Consequently, a separate ANOVA was conducted for each dependent variable, with each ANOVA evaluated at an alpha level of 0.05. Looking at the aqueous extracts (W) obtained by five minutes extraction, there was a high significance difference ($p<0.01$) between the values within each dependent variable considered, which confirms the influence of microwave power on the extraction process.

As reported in Table 4, a similar content of TPC and TF was observed for the extractions carried out at 250 and 500 Watt for 5 minutes, as well as for the expression of antioxidant activity by DPPH assay ($p>0.05$). However, a reversal was noted when the applied microwave power was increased up to 800 Watt, observing a significant decline in all the studied parameters.

By increasing the microwave power over 500 Watt for 5 minutes extraction, the recovery of polyphenols and flavonoids decreased: these results agree with those reported by Shao et al. [36]. A different trend was found for the 15 minutes extractions, among which there was no significant difference in terms of TPC regardless of the power applied, as well as for the results of DPPH assay. In contrast to this, a highly significant difference ($p<0.01$) was determined with regards to the extraction rate of flavonoid compounds and the relative expression of antioxidant activity by ABTS assay, even if without a positive correlation ($r=-0.78$). In this case, although water is a highly

polar solvent for the extraction of bioactive compounds, the main inconvenience of its use is the difficulty of determining the high content of water-soluble impurities which interfere with the identification and quantification of target compounds [8].

Despite a certain correlation between increasing of time and extraction yield was found, few minutes up to 500 Watt of microwave treatment seemed to show the best extraction yield in polyphenols and flavonoids: an overexposure in the microwave encouraged in fact the degradation of the thermolabile compounds, as reported by Nayak et al. [37]. According to Rafiee et al. [34], the increase in irradiation time up to 15 minutes did not result in improvement in the extraction performance, but sometimes might lead to a decrease in the concentration yield.

About the hydroalcoholic extracts (W/EtOH), by increasing the microwave power from 250 to 500 Watt with extraction time fixed at 5 minutes, the TPC decreased significantly from 6.74±0.22 to 5.56±0.30 mg GAE g^{-1} d.w. The reduction in TPC was noticed when the optimal conditions determined within the range of variables considered in this study have been exceeded (beyond 250 W and 5 minutes). This result was due to the increment in direct effect of microwave energy on the extraction system by the dipolar rotation, which resulted in temperature increase of the system and produced the deterioration of the bioactive substances [37].

Results indicated that the content of phenolic and flavonoid compounds decreased significantly with power during microwave treatment ($p<0.05$) and extraction time of 15 minutes did not show any obvious positive effect on extraction yield. In fact, when lemon by-products were treated at 250 Watt for 15 min there was a sudden decrease of value, compared with the extraction at 250 Watt for 5 minutes, which indicated that longer irradiation time was harmful to the TPC. This suggests that a shorter treatment time is ideal for the release of TPC under microwave irradiation. The highest content of total phenolic content and total flavonoids content was obtained when dried lemon by-products were treated at 250 Watt for 5 minutes, with aqueous-ethanol mixture as extraction solvent. The results suggested that the interaction between the extraction solvent (W or W/EtOH) and microwave power was highly significant ($p<0.01$) on the extraction efficiency of TPC. Water

content, being the most common absorbing phase for microwave energy, shows a crucial role during microwave extraction process [38]. Phenolic compounds are polar molecules and the presence of water boosts the polarity index of organic solvents compared with pure solvents, as ethanol, contributing to get higher values for the TPC and antioxidant activity. Moreover, the addition of water not only increases the dielectric constant of the solvent, but also intensifies absorption of microwave energy provoking a higher temperature inside the samples, which causes the fracture of cells and a quick outflow of antioxidant compounds [13]. Prolonged extraction time and ~~a~~ high microwave power led to lower TPC values, as a result of the almost certain~~ly~~ damage to phenolic acids by microwave treatment [38, 39].

Regarding antioxidant capacity of aqueous extracts (W), the same trend of the valuable compounds previously discussed was observed, increasing slightly as microwave power increases up to 500 Watt, even if there is no significant difference between the values. After 5 minutes of extraction, the antioxidant activity of aqueous extracts performed by DPPH and ABTS assays was 5.44±0.37 and 8.61±0.51 μM TE g^{-1} d.w., respectively.

Highest antioxidant capacity of hydroalcoholic extracts (W/EtOH), in both DPPH and ABTS assays, was found after a microwave treatment at 250 Watt for 5 minutes. The expression of antioxidant activity, through both assays, perfectly reflects the values of the reported TPC and TF: correlation coefficients, in each case, were above 0.86, which meant that the increase in the antioxidant activity of the extracts was at least in part due to the increase in extracted valuable compounds. In general, the higher radical scavenging activity of MAE extracts could be explained by the fact that microwave treatment might affect cellular structure due to the sudden and swift increase in temperature and internal pressure, provoking a direct effect of microwaves on molecules, which results in rapid rise of the temperature and fast completion of a reaction [37]. In agreement with other authors, MAE could be identified as a fast and reliable method for bioactive compounds extraction from citrus wastes and by-products [2, 39].

Microwave extractive technique has proved to be more efficient in terms of time saving, with an extraction rate over 6 times faster than conventional and ultrasound assisted extraction. Indeed, MAE is characterised by physical and chemical phenomena that are basically distinct from those of the other two applied extraction techniques. The developed microwave system suggests that this kind of extraction offers clear advantages, such as less time required for the extraction process, as well as being environmentally safe.

Comparison of Conventional, Ultrasound (UAE) and Microwave Assisted Extraction (MAE)

In Figure 2 the results related to the TPC and TF extraction yield to all the tested samples were reported. It is clearly noticeable that the best extraction solvent used~~,~~ was the W/EtOH mixture, for all the applied extraction systems (conventional, ultrasounds-assistant and microwave-assistant). The TPC extraction yield ranged between: 19.47 and 40.13 mg GAE g^{-1} d.w. (conventional); 26.49 and 39.56 mg GAE g^{-1} d.w. (UAE) and 31.37 and 38.48 mg GAE g^{-1} d.w. (MAE). Regarding total flavonoids, the highest yield was also revealed for hydroalcoholic extracts, particularly in samples subjected to ultrasound-assisted extraction. All these results confirmed that the hydroalcoholic solvent has been found to be the best.

The best obtained extracts were compared among them and the main antioxidant properties were presented in Figure 3.

By comparing the various extraction techniques, the best antioxidant extracts obtained were selected~~,~~ not only with reference to the total content of bioactive compounds and related antioxidant activity, but also from the point of view of the cost-effectiveness of the extraction process, in terms of time saving. Indeed, choice of an extraction method would mostly depend on the advantages and disadvantages of the process such as extraction yield, complexity, production cost, time saving, environmental friendliness and safety [39]. In this study, for each kind of extraction, the following

optimal conditions were identified: 30 minutes at 70°C (conventional), 30 minutes at 70°C (UAE) and 5 minutes at 250 Watt (MAE).

Phenolic characterisation of the selected extracts obtained under optimal extraction conditions was performed by UHPLC system (Table 6, Fig.4). The method was developed by the injection of standard solutions at concentration levels. Coefficient of correlation (R^2), regression equations and limits of detection (LOD) and quantification (LOQ) for each antioxidant compound were reported in Table 5.

Eleven antioxidant compounds were identified in UAE extract: the chromatographic profile showed different concentrations of individual antioxidant compounds, with hesperidin (1694.98±0.36 mg kg^{-1} d.w.) and eriocitrin (1167.28± 0.25 mg kg^{-1} d.w.) as the most abundant flavonoids followed far away by narirutin and ferulic acid. Neohesperidin, naringin and rutin were found in very low amounts when compared to the other flavonoids. Apigenin was the least quantified antioxidant compound. Compared to conventional extraction, UAE allowed a better and more efficient extraction of these bioactive compounds. Indeed, as reported in Figure 2, it can be observed that a 30-minute UAE at 70°C extracted an equal amount of TPC and TF as a conventional 120-minute extraction at the same temperature.

As shown in Table 6, among all, UAE proved to be the best method that resulted in higher extraction yield of antioxidant compounds from lemon by-products, which was significantly higher than those of conventional and MAE. Even if UAE showed the best effect on the qualitative and quantitative characteristics of extracted bioactive compounds, it is a time-consuming method compared to the shortest process time (5 minutes) of microwave assisted extraction. MAE proved to be an interesting alternative extraction technique for the recovery of antioxidant compounds from lemon by-products through an environmentally green approach. The extraction time required for optimal recovery of antioxidant compounds was significantly less than that required for the other applied techniques, showing to be a more rapid extraction method.

In addition, the best selected extracts were subjected to antimicrobial activity analysis. Conventional and UAE extracts showed clear antimicrobial activity against some foodborne pathogens compared to the MAE extract (Table 7, Fig.5). The results of antibacterial activity are often highly correlated to total phenolic content. The results suggest that the bacteria species tested did not show an extremely sensitivity to the lemon by-products extracts, especially S. enterica, showing a halo diameter less or slightly more than 6 mm [40].

The antimicrobial properties of plant extracts are due to the presence of secondary metabolites, such as tannins, flavonoids and phenolic compounds, which are the most relevant active elements against bacteria [41]. The absence of antimicrobial activity in the MAE extract might be due to the lack of some valuable compounds, such as apigenin. Indeed, as reported by [42], apigenin has the capability to inhibit the growth of L. monocytogenes, thanks to its intense antibacterial activity by deactivating microbial adhesion, enzymes and cell transport proteins. A similar effect was detected by [43], who reported the contribution of gallic acid in the expression of antimicrobial activity against E. coli. As described by the author, the method of biologically active substances extraction is relevant to the final composition of the extract. In general, the UAE was found to be the best in terms of phenolic characterisation and antimicrobial activity.

Conclusion

In this study, the optimal extraction yield and antioxidant activity values were obtained when the extraction process was performed with the hydroalcoholic solvent (W/EtOH, 50%) for all the techniques applied. The comparative studies revealed that the recovery of antioxidant compounds from lemon by-products using UAE at 70°C for 30 minutes was significantly higher than those of conventional and microwave extraction.

Anyway, results obtained by MAE revealed it can be considered a novel time-consuming and high-efficient method in the extraction of many bioactive compounds from various natural matrices.

The studied extraction procedures involved an environmentally green approach and it could be generalized to other by-products to obtain antioxidant extracts for the future utilization as ingredients in functional food preparations.

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Figure caption

Figure 1: Simplified outline of the experimental procedures.

Figure 2: The effect of extraction method on total polyphenol yield (TPC yield) and total flavonoid yield (TF yield) of the extracts (a: conventional solid-liquid extraction, b: UAE, ultrasound assisted-extraction, c: MAE, microwave assisted extraction).

Figure 3: Main antioxidant results of the best extracts. The data are presented as means ± SD (n=4). TPC: total phenolic content; TF: total flavonoid content; DPPH and ABTS: antioxidant activity assays.
C (30', 70°C), conventional solid-liquid extraction for 30 minutes at 70°C; C (120', 70°C), conventional solid-liquid extraction for 120 minutes at 70°C; UAE (30', 70°C), ultrasound-assisted extraction for 30 minutes at 70°C; UAE (120', 70°C), ultrasound-assisted extraction for 120 minutes at 70°C; MAE (5', 250W), microwave-assisted extraction for 5 minutes at 250W.

Figure 4: Eexample of UHPLC analysis of individual antioxidant compounds in UAE extract (Ultrasound-assisted extraction at 70°C for 30 minutes). Peak designation was: (1) gallic acid, (2) caffeic acid, (3) p-cumaric acid, (4) ferulic acid, (5) rutin, (6) eriocitrin, (7) narirutin, (8) hesperidin, (9) neohesperidin, (10) naringin, (11) apigenin.

Figure 5: Antimicrobial activity results of the best extracts. Conventional: solid-liquid extraction at 70°C for 30 minutes. UAE: Ultrasound-assisted extraction at 70°C for 30 minutes. MAE: microwave-assisted extraction at 250W for 5 minutes. Control: water-ethanol 50% solution.

Table 1: Multivariate statistical analysis of lemon by-product extracts.

Conventional	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Temperature	**	**	**	**
Time	ns	**	ns	ns
Solvent*Temperature	**	**	**	**
Solvent*Time	**	**	**	**
Time*Temperature	**	**	*	*
Solvent*Temperature*Time	**	**	**	ns
UAE	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Temperature	**	**	**	**
Time	**	**	**	**
Solvent*Temperature	**	**	**	**
Solvent*Time	**	**	**	**
Time*Temperature	**	**	*	**
Solvent*Temperature*Time	**	**	*	ns
MAE	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Power	**	ns	*	**
Time	ns	**	ns	*
Solvent*Power	**	**	**	*
Solvent*Time	ns	ns	ns	ns
Time*Power	**	**	ns	*

Abbreviation: ns, not significant; ** Significance at $p<0.01$; * Significance at $p<0.05$.
Conventional: solid-liquid extraction; UAE: Ultrasound-assisted extraction; MAE: microwave-assisted extraction.
TPC: total phenolic content; TF: total flavonoid content; DPPH and ABTS: antioxidant activity assays.

Table 2: Total phenolic content (TPC), Total flavonoid content (TF) and antioxidant activity (DPPH and ABTS assays) values of conventional solid-liquid extraction (W, water, and W/EtOH, hydroalcoholic mixture).

		TPC (mg GAE g^{-1}d.w.)			TF (mg CE g^{-1}d.w.)		
Minutes	**°C**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
30	**25**	2.96±0.11^{a}	3.41±0.10^{c}	**	0.94±0.04^{ab}	0.61±0.04^{c}	**
	40	2.52±0.28^{b}	5.42±0.17^{b}	**	0.89±0.03^{b}	1.43±0.04^{b}	**
	70	2.69±0.02^{ab}	6.37±0.22^{a}	**	1.07±0.06^{a}	1.84±0.10^{a}	**
	Sign.	*	**		**	**	
60	**25**	2.24±0,06^{a}	4.92±0.09^{c}	**	0.31±0,06^{b}	1.11±0.05^{c}	**
	40	1.09±0.21^{b}	6.23±0.24^{b}	**	0.49±0.02^{a}	1.58±0.09^{b}	**
	70	1.35±0.58^{b}	6.75±0.25^{a}	**	0.30±0.00^{b}	1.85±0.07^{a}	**
	Sign.	**	**		**	**	
120	**25**	1.23±0.06	5.01±0.13^{b}	**	0.25±0.03 ^{b}	1.12±0.04^{c}	**
	40	1.48±0.48	6.86±0.17^{a}	**	0.38±0.05^{ab}	1.85±0.10^{b}	**
	70	1.93±0.60	7.03±0.48^{a}	**	0.58±0.24 ^{a}	2.22±0.29^{a}	**
	Sign.	**ns**	**		*	**	
		ABTS (µM TE g^{-1}d.w.)			**DPPH (µM TE g^{-1}d.w.)**		
Minutes	**°C**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
30	**25**	7.65±0.74	14.13±0.79^{b}	**	4.46±0.85	5.13±0.30^{b}	**ns**
	40	6.72±0.63	20.72±1.82^{a}	**	4.48±0.21	6.75±0.09^{a}	**
	70	7.52±0.57	19.43±0.95^{a}	**	4.22±0.11	6.96±0.24^{a}	**
	Sign.	**ns**	**		**ns**	**	
60	**25**	7.28±1.66	17.02±0.77^{b}	**	4.06±0.46	6.77±0.43	**
	40	6.78±2.32	19.11±0.60^{a}	**	3.61±1.30	7.23±0.11	**
	70	5.54±1.57	19.47±0.70^{a}	**	3.25±1.24	7.08±0.18	**
	Sign.	**ns**	**		**ns**	**ns**	
120	**25**	5.96±0.24	17.46±0.19^{c}	**	3.30±0.67	6.80±0.22^{b}	**
	40	6.03±0.74	21.50±0.76^{a}	**	3.31±0.73	7.76 ±0.51^{a}	**
	70	7.45±1.34	19.33±0.89^{b}	**	3.82±0.69	8.27±0.13^{a}	**
	Sign.	**ns**	**		**ns**	**	

The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; ** Significance at $p<0.01$; *Significance at $p<0.05$.

Table 3: Total phenolic content (TPC), Total flavonoid content (TF) ~~values~~ and antioxidant activity (DPPH and ABS assays) values of Ultrasound assisted extraction (W, water, and W/EtOH, hydroalcoholic mixture).

		TPC (mg GAE g^{-1} d.w.)			TF (mg CE g^{-1} d.w.)		
Minutes	**°C**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
30	**25**	5.13±0.10^{b}	4.64±0.13^{c}	**	1.47±0.09^{a}	1.17±0.04^{c}	**
	40	5.38±0.06^{a}	5.82±0.26^{b}	*	1.55±0.15^{a}	1.77±0.11^{b}	**ns**
	70	4.14±0.08^{c}	6.93±0.32^{a}	**	1.21±0.04^{b}	2.07±0.11^{a}	**
	Sign.	**	**		**	**	
60	**25**	5.91±0.20^{a}	6.75±0.34	**	2.30±0.08^{a}	2.04±0.09^{c}	**
	40	3.93±0.19^{b}	6.85±0.21	**	1.59±0.07^{b}	2.23±0.04^{b}	**
	70	2.61±0.06^{c}	6.73±0.21	**	1.03±0.02^{c}	2.40 0.09^{a}	**
	Sign.	**	**ns**		**	**	
120	**25**	4.27±0.12^{a}	6.30±0.15^{b}	**	0.95±0.05^{a}	1.97±0.07	**
	40	4.39±0.08^{a}	6.90±0.28^{a}	**	1.01±0.05^{a}	1.98±0.10	**
	70	1.31±0.04^{b}	6.93±0.15^{a}	**	0.25±0.05^{b}	2.13±0.12	**
	Sign.	**	**		**	**ns**	
		ABTS (µM TE g^{-1}d.w.)			**DPPH (µM TE g^{-1}d.w.)**		
Minutes	**°C**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
30	**25**	18.67±1.10	14.06±0.02^{c}	**	6.38±0.22^{a}	6.40±0.16^{c}	**ns**
	40	17.73±1.02	16.37±0.83^{b}	**ns**	6.16±0.15^{a}	7.13±0.13^{b}	**
	70	16.24±2.12	18.36±0.86^{a}	**ns**	5.03±0.58^{b}	7.43±0.09^{a}	**
	Sign.	**ns**	**		**	**	
60	**25**	10.24±0.94^{a}	19.42±0.63	**	6.26±0.28^{a}	8.25±0.24	**
	40	9.16±0.40^{a}	19.77±0.67	**	5.22±0.62^{b}	7.90±0.94	**
	70	7.03±0.67^{b}	19.62±1.06	**	3.86±0.31^{c}	7.95±0.95	**
	Sign.	**	**ns**		**	**ns**	
120	**25**	9.00±0.53^{a}	19.60±0.46^{b}	**	3.58±0.09^{a}	7.14±0.45	**
	40	5.91±0.34^{b}	20.41±0.55^{ab}	**	3.43±0.16^{a}	7.61±0.24	**
	70	3.07±0.44^{c}	21.28±0.29^{a}	**	2.08±0.21^{b}	7.59±0.20	**
	Sign.	**	**		**	**ns**	

The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; **Significance at $p<0.01$.

Table 4: Total phenolic content (TPC), Total flavonoid content (TF) ~~values~~ and antioxidant activity (DPPH and ABS assays) values of Microwave-assisted extraction (W, water, and W/EtOH, hydroalcoholic mixture).

		TPC (mg GAE g^{-1}d.w.)			TF (mg CE g^{-1}d.w.)		
Minutes	**Watt**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
5	**250**	2.94±0.11[a]	6.74±0.22[a]	**	0.90±0.04[a]	2.02±0.13[a]	**
	500	3.28±0.27[a]	5.56±0.30[b]	**	0.98±0.09[a]	1.62±0.11[b]	**
	800	2.48±0.09[b]	5.56±0.27[b]	**	0.69±0.07[b]	1.75±0.13[b]	**
	Sign.	**	**		**	**	
	250	3.00±0.10	5.62±0.49	**	0.50±0.05[b]	1.59±0.28	**
	500	3.14±0.54	5.87±0.22	**	0,75±0.08[a]	1.74±0.12	**
15	**800**	2.56±0.11	5.63±0.19	**	0,89±0.10[a]	1.69±0.08	**
	Sign.	**ns**	**ns**		**	**ns**	
		ABTS (µM TE g^{-1}d.w.)			**DPPH (µM TE g^{-1}d.w.)**		
Minutes	**Watt**	**W**	**W/EtOH**	**Sign.**	**W**	**W/EtOH**	**Sign.**
5	**250**	7.45±0.50[b]	24.08±1.01[a]	**	5.33±0.46[a]	12.01±0,30[a]	**
	500	8.61±0.51[a]	20.03±0.94[b]	**	5.44±0.37[a]	10.55±0.40[b]	**
	800	6.87±0.66[b]	21.35±1.09[b]	**	4.37±0.22[b]	10.72±0.19[b]	**
	Sign.	**	**		**	**	
	250	7.96±0.33[a]	21.56±0.81	**	5.05±0.27	10.81±0.36	**
	500	7.91±0.66[a]	21.73±1.94	**	5.06±0.75	10.82±0.31	**
15	**800**	6.37±0.29[b]	21.99±2.12	**	4.74±0.70	10.41±0.12	**
	Sign.	**	**ns**		**ns**	**ns**	

The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; ** Significance at $p<0.01$.

Table 5: Regression equation, Correlation coefficient (R^2), Limits of detection (LOD) and Limits of quantification (LOQ) in standard solutions detected by UHPLC.

Compounds	Regression equation	R^2	LOD mg kg^{-1}	LOQ mg kg^{-1}
Gallic acid	y=150.5x+48.18	0.9997	0.0921	0.7526
Caffeic acid	y=111.97x-84.67	0.9997	0.078	0.2567
***p*-Coumaric acid**	y=114.17x+35.47	0.9999	0.0812	0.3145
Ferulic acid	y=127.18x-72.81	0.9998	0.0774	0.5676
Rutin	y=46.07x-14.44	0.9994	0.09676	0.3209
Eriocitrin	y=41.392x-38.81	0.9994	0.3574	19.082
Narirutin	y=88.81+84.18	0.9998	0.08123	0.7654
Hesperidin	y=54.81x+24.38	0.9991	0.07675	23.452
Neohesperidin	y=115.12x+6.96	0.9999	0.0536	0.8657
Naringin	y=42.17x-20.75	0.9994	0.06756	0.5768
Apigenin	y=100.91x-296.57	0.9966	0.04567	0.2345

Table 6: Phenolic characterisation of selected hydroalcoholic lemon by-products extracts (mg kg^{-1} d.w.).

Compounds	Conventional	UAE	MAE	Sign.
Gallic acid	72.92±0.14^{b}	103.97±0.28^{a}	2.08±0.14^{c}	**
Caffeic acid	87.80±0.21^{a}	87.46±0.21^{a}	65.29±0.18^{b}	**
***p*-Coumaric acid**	59.25±0.23^{a}	58.17±0.29^{b}	57.81±0.19^{b}	**
Ferulic acid	134.86±0.24^{a}	134.48±0.32^{a}	85.73±0.19^{b}	**
Rutin	70.71±0.21^{b}	76.96±0.13^{a}	32.76±0.17^{c}	**
Eriocitrin	1129.06±0.22^{b}	1167.28±0.25^{a}	1001.34±0.24^{c}	**
Narirutin	58.50±0.17^{c}	140.89±0.24^{a}	77.21±0.25^{b}	**
Hesperidin	1636.26±0.26^{b}	1694.98±0.36^{a}	780.78±0.04^{c}	**
Neohesperidin	6.11±0.24^{b}	12.45±0.19^{a}	1.87±0.19^{c}	**
Naringin	33.39±0.22^{b}	44.14±0.22^{a}	19.84±0.21^{c}	**
Apigenin	3.43±0.26^{b}	4.29±0.08^{a}	nd	**

The data are presented as means ± SD (n=3). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: nd, not detected; Sign., significance; **Significance at $p<0.01$.

Conventional: solid-liquid extraction at 70°C for 30 minutes; UAE: Ultrasound-assisted extraction at 70°C for 30 minutes; MAE: microwave-assisted extraction at 250W for 5 minutes.

Table 7: Antimicrobial activity of selected hydroalcoholic lemon by-products extracts. Data are expressed as mm of the halo diameter resulting from the antimicrobial activity of the extract.

	L. monocytogenes **ATCC 13932**	*E. coli* **ATCC 8739**	*S. enterica* **wild**
Conventional	7.1±0.1	0.0	4.0± 0.0
UAE	7.0±0.0	6.6±0.14	4.0± 0.0
MAE	0.0	0.0	0.0
Control	0.0	0.0	0.0

The data are presented as means ± SD (n=2).
Conventional: solid-liquid extraction at 70°C for 30 minutes.
UAE: Ultrasound-assisted extraction at 70°C for 30 minutes.
MAE: microwave-assisted extraction at 250W for 5 minutes.
Control: water-ethanol 50% solution.