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# RECOVERY OF HESPERIDIN FROM LEMON BY-PRODUCTS USING GREEN TECHNIQUES

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**Abstract:** *The food industry is the main producer of food waste. Therefore, the development of methodologies to reuse and make the most of it is of great interest. Food industry waste can contain compounds with added value that, if properly extracted and used, can be applied to the development of healthy foods (clean label), nutraceuticals, senior food, cosmetics, etc. The revaluation of by-products from the citrus industry will make it possible to reduce the large volume of lemon waste, reducing the cost of waste management and obtaining compounds of interest from them. In this work, different alternative methodologies to the use of organic solvents have been used for the extraction of Hesperidin in lemon by-products, including thermal, enzymatic and ultrasound treatment and purification adsorption processes with resins, granular activated carbon and zeolite were optimized. The optimum Hesperidin extraction (86371 ± 422 mg GAE/g dry extract) was achieved using an enzymatic pre-treatment, cellulase enzyme (Validase TRL®-Sigma) at 0.01% of weight of the lemon peel by-product, treatment time of 60 min, and water/citrus by-product ratio of 3:1 w/w, followed by a purification adsorption/desorption process through Purolite PAD610 resin.*

**Keywords:** *Green techniques, citrus by-product, extraction, lemon extract, Ultrasound treatment, Enzymatic treatment, adsorption, desorption, phenolic compounds recovery.*

## 1. Introduction

For several decades, agro-industrial waste has been a focus of attention for researchers worldwide. Industrial and agricultural wastes are materials in solid or liquid state that are generated from the direct consumption of primary products or their industrialization, and that are no longer useful for the process that generated them, they are susceptible of use or transformation to generate another product with economic value, of commercial and/or social interest [1]. The valorisation of by-products of the food industry is presented as a sustainable alternative within the European framework, considering the serious environmental problem it causes, where 1.600 MT of waste generate 3.3 billion tons of $CO_2$ equivalent of greenhouse gases released into the atmosphere per year. [2].

The current need for a sustainable food chain requires the implementation of a circular economy approach in the processing industries. The main focus of this approach is to revalorize the discarded parts of vegetables due to their high content of bioactive compounds, that can be used for application in different industries, due to their antioxidant, antimicrobial and functional power. [3,4].

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Recently, food by-products such as citrus (genus Citrus L.) by-products have been studied for their content of other non-nutritional compounds with potential biological properties, such as flavonoids, carotenoids, vitamins, and minerals, which may help reduce the risk of many chronic diseases (cardiovascular and age-related degenerative diseases) [5].

The most abundant flavonoid in citrus has been identified as hesperidin [6] also well known for its antifungal activity [7] cardiovascular beneficial effects [8], antioxidant [9] and anticancer [10] properties. Numerous applications as food ingredient or preservative to extend shelf life, however, functional properties are also for its special consideration too. [11-12]

Citrus by-products are composed of about 50% by weight of matter, consisting of rind, seeds, pulp and peel [13].

Different studies use green methodologies for the extraction of flavonoids such us hesperidin from lemon residues, including the use of enzymes, thermal treatments and ultrasound technology [14].

With the appropriate enzymes it is possible to break the structure of the cell membranes to provoke the release of the citrus flavonoids such us Hesperidin. Essential oils have been extracted from citrus peels (orange and lemon) using enzymatic treatments with cellulases. The yield obtained showed an increase from 2 to 6 [15-17].

The ultrasound technology is based in cells disruption by cavitation. As a result, internal diffusion, and the transfer of compounds of interest from agri-food waste to extract is increased. The use of ultrasound-assisted techniques favours the extraction yield. The extraction of flavonoids from citrus peels was studied, reducing the extraction time and temperature when ultrasonic technology was used. [18-21].

Adsorption processes are an optimal technology for the recovery of bioactive compounds. This is due to the chemical affinity with compounds in the surface area, selectivity and the facility of regeneration of resins [22]. For the elution of compounds of interest, one of the best eluents is ethanol. [23, 24]

In this study, different green extraction and purification technologies, including the use of enzymes, thermal treatments and ultrasound technology, alternative to the conventional ones, were developed to obtain extract rich in Hesperidin and polyphenols from lemon by-products. The purification of the extract was carried out by adsorption-desorption processes with resins, granular activated carbon (GAC) and zeolite. Compounds of interest such as Hesperidin and total polyphenolic compounds were characterised in lemon extracts obtained.

## 2. Materials and methods

### *2.1 Plant material*

The lemon (*Citrus limon* (L.) Burm. F) by-products composed of lemon peels were supplied by several companies in Murcia- Spain along March and April of 2022. The lemon peels were transported directly to lab within no more than 1 hour after their generation and stored at -18 $\pm$ 1ºC until extraction experiments were conducted.

### *2.2 Adsorbent materials*

Granular activated carbon (GAC), particle ranging from 0.5-3.13 mm, was supplied by Zeocat (Barcelona, Spain)
As for zeolite adsorbent, with a 0.6-1.5 mm particle size, was supplied by Panreacapplichem (Barcelona, Spain). The polymethacrylic resin crosslinked with divinylbenzene (PAD610), polydivinybenzene resin (PAD900) and polymethacrylic resin (PAD950) were supplied by Purolite (Hangzhou, China). Table 1 shows the technical description of the adsorbent materials used.

**Table 1.**
**Physical properties of the adsorbents used (supplier's information).**

| Resin | Matrix | *Surface area ($m^2 \cdot g^{-1}$) | *Pore diameter (Å) | *Pore volume ($mg \cdot L^{-1}$) | Specify gravity | Particle size (mm) | Hydrophobicity |
|---|---|---|---|---|---|---|---|
| PAD610 | PMC-DVB[1] | 490 | 300 | 1.2 | 1.1 | 0.35-1.2 | Moderate |
| PAD900 | PDVB[2] | 850 | 220 | 1.9 | 1.02 | 0.35-1.2 | Moderate |
| PAD950 | Polymethacrylic | 450 | 120 | 0.6 | 1.1 | 0.35-1.2 | Moderate |
| ZEOLITE | Aluminosilicate | 70-80 | 4-8 | n.d | 0.85 | 0.6-1.5 | Low |
| CAG | Carbon | n.d | n.d | n.d | n.d | 0.5-3.13 | Low |

**Measured by nitrogen adsorption*
*[1] PMC-DVB: Polymethacrylics cross-linked with divinylbenzene*
*[2] PDVB: Polydivinylbenzene*
*n.d: not determined*

Elution of the compounds of interest adsorbed on the resin surface was carried out by desorption process with ethanol (EtOH) 96% Scharlau (Barcelona, Spain).

### *2.3 Experimental design*

In this study three techniques were applied to look into the impact of hesperidin and polyphenolic compounds. The best technology was then applied for further investigation to determinate the best flavonoids and hesperidin adsorbent materials with the aim to purify the extracts increasing the recovery yield.
Concentration of the hesperidin and total polyphenols in the extracts were compared for identification of suitable conditions for maximum recovery yield from lemon peels. The figure 1 shows the experimental design.

### *2.4 Extraction of phenolic compounds using different techniques.*

Extracts of interest were prepared with three different treatments as follow:
*Aqueous treatment (WE)*: Water was added to lemon by-product in a 3:1 (w/w) proportion. It was them heated up to 100ºC and constantly stirred for 60 minutes.
*Enzymatic treatment (EE)*: The lemon by-product was mixed with water 1:3 (w/w). A cellulase enzyme (Validase TRL®-Sigma) was added at 0.01% of weight of the lemon peel by-product and the mixture was kept stirred in constant agitation at 25 °C for 60 minutes. Then the enzyme was deactivated heating the mixture at 100 °C. This methodology was described by the enzyme supplier.

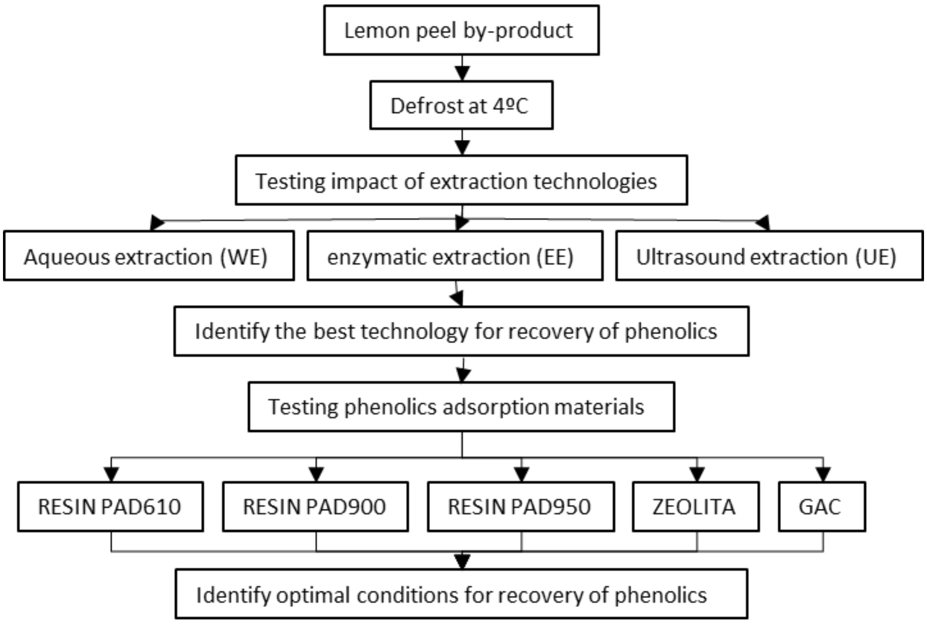


**Fig. 1. Experimental design**

*Ultrasound treatment* (US): According with the procedure described by Saifullah et al (2020) [25], the lemon by-product was mixed with water 1:3 (w/w).

An ultrasonic treatment in a UIP500hdT equipment (Hielscher- Germany) with a power of 164 W, amplitude of 100 % with constant agitation at 75 °C for 60 minutes was applied to the mixture.

All the samples obtained through the WE, EE and US treatment were centrifugated (Centrífuga Heraeus Labofuge 200, Thermo Scientific, Massachusetts, EEUU) at 11.200xg for 20 min, filtered (Whatman nº. 42) and lyophilised using Coolvacuum Lyoepic-85 (Coolvacuum Technologies, Granollers, Spain).

All the experimental processes were conducted on triplicate. These conditions were defined in preliminary tests, considering the sample characteristics and the limitation of the apparatus. Moreover, recommendations of Granone et col (2022) [26], Saifullah et al (2020) [25], SI-Yu Lu et al (2021) [27] and Menezes-Barbosa el al. [28] were followed.

### *2.5 Purification of the extract using different adsorbers materials.*

Batch adsorption experiments were carried out according to the methodology described by Drevelegka et al (2020) [29]. Prior to every experiment, the adsorber materials and resins were pretreated following the suppliers' recommendations. The lemon by-product was processed through the enzymatic treatment (EE), the liquid obtained after centrifugation and filtration steps (as described in point 2.4)

was mixed with 5 different adsorbates 10:1 (w/w): Resin PAD610 (Purolite), Resin PAD900 (Purolite), Resin PAD950 (Purolite), Zeolite (Zeocat) and Granular Activated Carbon (GAC) (PanReac AppliChem). It was kept in contact with the adsorbates under constant agitation for 30 minutes at room temperature.

After that the adsorbated hesperidin and polyphenols were eluted with EtOH 96% 1:1 (w/w).

Finally, the solvent was evaporated from the purified extracts in a vacuum evaporator (Evaporator R-200, Buchi, Switzerland).

All the experiments were carried out in triplicate.

### *2.6 Determination of Total Polyphenols*

The total polyphenols content was determined using Folin-Ciocalteu method. Folin-Ciocalteu reagent (FCR) was prepared from water (90 ml) and Folin-Ciocalteu stock solution (10 ml). A dilution was made by mixing with FCR (5 ml), 5% sodium carbonate solution (4 ml) and the dissolved sample and kept in the dark for 1 hour. After 1 hour, the sample was measured in a spectrophotometer (UVmini 1240 Shimadzu, Kuoto, Japan) with a wavelength of 765 nm. The units used to express the total polyphenols were mg gallic acid equivalents per 1 g dry matter (mg GAE/kg DM). [30].

### *2.7 Determination of Limonin*

Limonin was analysed on a liquid chromatography HPLC system with detector photodiode array detector PAD (Agilent 1100, Santa Clara, EEUU): wavelength 207 nm, with a mobile phase A of 55% water and a mobile phase B of 45% ACN, a flow rate of 1 mL/min, an injection volume of 20mL, a C-18 column with a temperature of 45 °C according to Can Liu et al. [31]

### *2.8 Determination of Hesperidin*

Hesperidin was analysed on a HPLC systems equipped with a PDA (photodiode array detector) (Agilent 1100, Santa Clara, EEUU) with fixed wavelength at 260 nm. The mobile phase that was used consisted of two solvents: water (A) and acetonitrile (B), both containing 0.1% acetic acid (v/v).

For elution, a gradient elution with a sample injection volume of 5 µL was used with a Kinetex C18 column (100 × 4.6 mm, 2.6 µm, 100 Å, Phenomenex Torrance, CA, USA) with the following parameters: 1 min (10% B), 2 min (20% B), 4 min (30% B), 5 min (90% B), 8 min (10% B) at a temperature of 52 °C with a mobile phase flow rate of 1.0 mL/min.

The parameters used were proposed by Chaves et al [32].

### *2.9 Data processing*

The experiments were performed in triplicate as were the analytical determinations. The values presented are the mean values obtained together with the standard deviation (SD).

## 3. Results and Discussion

*Effect of the WE, EE and US treatments in the extraction of Hesperidin and Polyphenols from the lemon peels.*

Table 2 reported the values of hesperidin and polyphenols in the three lyophilized lemon extracts. Table 2 illustrated the concentration of compounds of interest.: hesperidin and polyphenols. Highlighting that the highest concentration of Hesperidin (17689.5 mg/kg) and the total polyphenols (59606.1 mg GAE/kg) were obtained when the EE treatment was used. Compared to the other extractions techniques (WE and US), EE produces an extract of higher quality in shorter processing times [33].

**Table 2.**

**Effect of WE, EE and US treatment in the extraction of hesperidin and polyphenols for the lemon peels.**

| | WE | EE | US |
|---|---|---|---|
| **HESPERIDIN, (mg/kg)** | 14676.9 ± 164 | 17689.5 ± 187 | 9377.3 ± 85 |
| **TOTAL POLYPHENOLS, (mg GAE/Kg DM)** | 23936.9 ± 254 | 59606.1 ± 425 | 22816.6 ± 241 |

*Results are expressed as mean values of triplicates ± S.D.*
*WE: Aqueous treatment*
*EE: Enzymatic treatment*
*US: Ultrasound treatment*

Several experiments have used enzymes to increase the concentration of polyphenols in various vegetables, including citrus fruits [34]. Enzymes can be used with other extraction processes, such as ultrasound, thereby increasing the yield of polyphenols and making the processes more cost-effective. [35].

*Purification of hesperidin and polyphenols from lemon peel by adsorption/desorption process.*

The enzymatic treatment was found to be the most profitable technique for the recovery of hesperidin and polyphenols. It was chosen as a pre-treatment for further purification through adsorption/desorption process. So as to increase hesperidin and polyphenols concentration in lemon extracts, the efficiency of several adsorbent materials were compared. After performing the purification procedure as described in the apart 2.5, the concentration of hesperidin and polyphenols were determined for each dry extract obtained.

Table 3 shows the results of the analysis of the hesperidin and polyphenols after the purification of the liquid obtained after the treatment of the lemon peel with cellulose with different adsorbent materials.

The best adsorbent for obtaining an extract with a high concentration of total polyphenols was the resin PAD900, this effect may be due to the higher affinity of the polyphenols for the surface of this resin [36]. Cifuentes-Cabezas et al. [37] investigated the application of MN200, MN202 PAD900 and PAD950 for polyphenol retention using olive mill wastewater and found that the most optimal resin for this application was PAD900 resin.

The adsorbent material with the best performance for obtaining extract rich in Hesperidin was Resin PAD610, with the highest pore size (table 1), resulted more efficient for the adsorption of compounds with a big structure like hesperidin. When Zeolite and CAG were used in the adsorption processes, low concentrations of Hesperidin and total polyphenols were obtained because citrus polyphenols have a strong hydrophilic characteristic [38-39].

**Table 3.**

**Concentration of hesperidin and polyphenols in purified lemon extracts.**

| | RESIN PAD610 | RESIN PAD900 | RESIN PAD950 | ZEOLITA | GAC |
|---|---|---|---|---|---|
| **Determination of Compounds Interest** | | | | | |
| **HESPERIDIN, (mg/kg DM)** | 86371.2 ± 422 | 33912.9 ± 205 | 31552.8 ± 251 | 18300.5 ± 116 | 6200.8 ± 74 |
| **TOTAL POLYPHENOL, (mg GAE/Kg DM)** | 136670.2 ± 712 | 152616.8 ± 781 | 142728.1 ± 765 | 61752.9 ± 463 | 28722.3 ± 297 |

*Results are expressed as mean values of triplicates ± S.D.*

## 4. Conclusions

In the present work, the efficiency of treatment with different technologies for the extraction of hesperidin and polyphenols from lemon waste was studied. The technologies were based on water heat treatment, enzymatic treatment and ultrasound treatment. The enzymatic treatment with cellulose was showed the best results in terms of recovery hesperidin and polyphenols. In addition, extracts with a high concentration of hesperidin and polyphenolic compounds were obtained through adsorption-desorption processes as a purification method with resins.

The resins tested were showed good affinity for the citrus phenolic compounds, presenting high percentages of adsorption/desorption. The main conclusion is extract rich in hesperidin compound of high purity could be obtained.

In summary, this work has shown an optimized methodology to revalue lemon peels by-products using enzymatic technology as a treatment.

Regarding the procedure cellulase at a concentration of 0.01 % w/w was first added, following with the addition of water in a proportion of 3/1 (w/w) and , time of extraction of 60 min.

After enzymatic extraction, the hesperidin and the polyphenols were purified by divinylbenzene resin (PAD610 - Purolite), with an optimum hesperidin concentration of 86371.2 ± 422 mg/kg DM.

A strategy applied to the integral use of food lemon by-products based on obtaining bioactive substances of interest was proposed. To this end, different environmentally friendly methodologies that require a reduced consumption of solvents and energy have been researched. These applied treatments are consider as green extraction, as it eliminates the use of environmentally harmful organic solvents, uses natural resources that companies have as waste and a final product with a quality concentration of interest compounds is obtained. In future research we will optimize the process conditions to industrial scale up.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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