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Chemical and Biological Characterisation of Orange (*Citrus sinensis*) Peel Extracts Obtained by Subcritical Water

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Abstract: Orange peels, generally considered as waste, were treated with subcritical water (SWE)—a green technology and environmentally friendly extraction process—at different temperatures (120–200 °C) and extraction times (5–60 min). The extracts which were obtained were chemically and biologically characterised to evaluate this potential source of bioactive compounds. The extracts total phenolics content (TPC) and total flavonoids contents (TFC), as well as total antioxidant capacity (TAC), DPPH radical scavenging activity, and total carbohydrate content, were determined by UV spectrophotometry. The pectin content was quantified by a gravimetric method. The dietary fibre content was investigated, and a phytochemical screening assay was performed. The extract obtained at 120 °C for 5 min displayed the highest TPC (45.45 mg GAE/g DW), TFC (9.29 mg RE/g DW), and TAC (130.47 mg AAE/g DW), indicating that relatively low temperatures and extremely short extraction times can be used in SWE to obtain orange peel extracts that are rich in bioactive compounds. The results of this study demonstrate the exceptional potential of orange peel extracts obtained with SWE. As shown, this biowaste represents a promising source of health-promoting compounds that could be used in pharmaceutical and dietary products.

Keywords: sweet orange peel; subcritical water extraction; biological and chemical characterisation

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1. Introduction

The total global production of citrus fruits is over 124 million tonnes per year [1–3], which is mainly consumed fresh or in the form of juice worldwide. One third of all citrus fruit is processed, and thousands of tonnes of peels and other waste (pulp, seeds, damaged fruit, or fruit that does not meet quality standards) produced during citrus processing are commonly considered as agro-industrial waste [3,4]. Sweet oranges (*Citrus sinensis*) account for about 70% of total citrus production and consumption [5]. Their processing generates more than 16 million tonnes of waste annually [6], which is about 30–40% by weight of the processed fruit. About 60–65% of this waste is peel, while 30–35% is pulp and seeds [7]. If these materials are not further processed, they are discarded without meaningful reuse, which usually results in significant environmental pollution [8,9].

However, a variety of uses have been found for citrus peels, such as energy production through combustion [10], biogas production [11], or their use as promising biofuels with bioethanol and methane as end products [10]. Citrus peel waste can also be used for composting or as an ingredient in livestock feed [6,9].

Sweet orange peels, as well as citrus peels in general, are excellent sources of natural bioactive compounds: essential oils, polyphenols, fibres, minerals, pectin, and monosaccharides [12]. The essential oils range from 0.4 to 0.5% and contain different types of terpenes, with limonene being the main component (90–95%), as well as oxygenated compounds, namely alcohols, aldehydes, and esters [6]. Polyphenolic compounds, which are good

antioxidants and anti-inflammatory agents, are usually present in orange peels in amounts ranging from 8 to 32 g gallic acid equivalent (GAE) per kg, mainly in the form of phenolic acids (from the cinnamic acid family, usually bound to carbohydrate components), flavonoids, and their derivatives [6]. In addition, citrus peels are rich in sugars, either as free monosaccharides and disaccharides (glucose, fructose, and sucrose in a ratio of 35–36%) or polymerised to cellulose (glucose), hemicellulose (galactose, arabinose, and xylose) and pectin (rhamnose). Pectin is a heteropolysaccharide of 80–400 kDa with a high content of galacturonic acid. It is a valuable by-product of citrus peels that has a high nutraceutical value and is used in the food, pharmaceutical, and cosmetic industries as a gelling and thickening agent, and as a stabiliser [6,13]. The presence of such valuable biologically active components shows that orange peels can be used as rich natural resources [9].

To better characterise and quantify the active compounds, it is important to choose an effective and appropriate extraction technique [14]. Many factors can influence the extraction process, including the properties of the matrix, the solvent, the temperature, the pressure, the time applied, and the solvent to matrix ratio.

In recent years, new technologies and their combined and hybrid techniques have been explored to improve extraction, while reducing the environmental impact and energy consumption: ultrasound-assisted extraction, microwave-assisted extraction, pulsed electric field extraction, supercritical fluid extraction, and subcritical water extraction, to name a few [6,7].

Subcritical water extraction (SWE) is an environmentally friendly technique, as it uses water as the extraction medium, which is a non-toxic, non-flammable, and environmentally friendly solvent and does not produce greenhouse gases or waste. In SWE, water is used in its subcritical state—at temperatures and pressures below its critical points (Tc = 374.15 °C; Pc = 22.1 MPa). When the temperature and pressure change, the physicochemical properties (especially the dielectric constant) of water also change, which enables the selective extraction of polar, medium-polar, weak-polar, and non-polar compounds. Temperature is the most important parameter in the extraction process, while the pressure must be high enough to keep the water in its liquid state. High temperatures can cause the degradation of some heat-sensitive compounds. However, the degradation of extract constituents is not only dependent on the temperature, but also on the exposure time, which can influence the extraction efficiency [15]. In addition, subcritical water can be more reactive and corrosive than ambient water and can catalyse or accelerate the hydrolysis, oxidation, and other degradation pathways of some constituents [14].

As far as we know, there are very few studies in the scientific literature on the SWE of phytochemicals from orange peel. Lachos-Perez et al. [15] investigated the effects of operating parameters (temperature and water flow rate in a continuous process) on the SWE of flavonoids from defatted orange peels. In another study, the same research group [16] evaluated a two-step hydrothermal process for the sequential removal of flavanones at 150 °C in the first step and the hydrolysis of the residual biomass at temperatures above 200 °C in the second step to obtain monosaccharides and disaccharides.

The aim of this study was to shed new light on the phytochemicals in orange peels, to characterise the extracts obtained by SWE as a green, environmentally friendly, and safe technology at different temperatures and extraction times, and to compare them in terms of their properties. The biological and chemical characterisation of the extracts was done by determining the TPC (total phenolics content), TFC (total flavonoids content), total antioxidant capacity (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total carbohydrate content (TCC), pectin content, dietary fibre content (DFC), and phytochemical screening assay. The novelty of the presented work consists of five orange peel extracts with different biological and chemical properties and potential use in the pharmaceutical, cosmetic, and dietary industries.

2. Materials and Methods

2.1. Plant Material

Commercially available Spanish sweet oranges were purchased from a local market in Novi Sad, Serbia, in January 2022. The oranges were washed and peeled. The peels (albedo and flavedo) were air-dried at room temperature (about 24 °C) in dark until constant weight. The dried orange peels were ground with a laboratory blender and stored in a glass jar at room temperature until extraction.

2.2. Chemicals and Reagents

Gallic acid and rutin trihydrate were purchased from Dr. Ehrenstorfer GmbH (Ausburg, Germany). Ascorbic acid, glucose, and DPPH were purchased from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent was purchased from Lachner (Neratovice, Czech Republic). Sodium carbonate and aluminium chloride hexahydrate were purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Hydrochloric acid and sulphuric acid were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium phosphate dodecahydrate and ammonium molibdate tetrahydrate were purchased from Centrohem (Stara Pazova, Serbia). Phenol, methanol, and ethanol were purchased from Zorka (Šabac, Serbia). Nitrogen under pressure (99.999%) was supplied by Messer (Bad Soden, Germany). All other chemicals were of analytical reagent grade.

2.3. Subcritical Water Extraction

SWE of orange peels was carried out in a homemade subcritical water extractor/reactor with a high-pressure stainless steel process vessel with a total capacity of 1.7 litres [17]. The plant material and distilled water were put into the reaction vessel in a ratio of 1:20 (w/w). After closing, the extraction cell was pressurised with nitrogen through the gas inlet valve installed in the lid of the reaction vessel. The pressure was kept constant (15 bar) during the extraction process. The reaction vessel was placed on a heating/vibration platform. The heating rate was about 10 °C/min and the frequency of the vibration was kept at 3 Hz. After the operating temperature (120–200 °C), with the temperature being controlled by a digital temperature controller, was reached, the time (5–60 min) was measured. After extraction, the process vessel was cooled to 20 ± 2 °C in a flow-through water bath and the pressure was released by opening the valve. The extracts obtained were filtered through a Whatman filter paper, grade 1, and stored in polyethylene bottles in the refrigerator (4 °C) for further analysis.

2.4. Total Extraction Yield

Extracts of orange peel of a certain volume (2 mL) were evaporated to dryness by heating at 60 °C to constant weight (about 24 h). The extraction yield was calculated as g of dry extract per 100 g of dry orange peels (g extract/100 g DW).

2.5. Total Phenolics Content (TPC)

The total phenolics content of orange peel extracts from subcritical water was measured using the Folin–Ciocalteu method [18]. The reaction mixture was prepared by mixing 400 µL of the extract or standard solution with 2 mL of diluted Folin–Ciocalteu reagent (1:10, v/v) and adding 1.6 mL of sodium carbonate solution (7.5%, w/w) after 4 min. The blank was prepared using distilled water instead of the extracts. The mixtures were incubated at room temperature for 90 min for colour development, and absorbance was measured at 765 nm. The TPC was calculated by interpolating the measured sample absorbance into a calibration curve defined using standard solutions of gallic acid for the 0–200 mg/L concentration range ($A = 0.0108\gamma + 0.0134$, $r^2 = 0.9996$). Measurements were performed in triplicate for each sample. Results were expressed as mg gallic acid equivalent per gramme of orange peel dry weight (mg GAE/g DW) and calculated as mean ± SD.

2.6. Total Flavonoids Content (TFC)

The total flavonoids content of the orange peel extracts was determined by a colorimetric method using $AlCl_3$ [19]. Briefly, 2 mL of a 2% $AlCl_3$ solution was added to 2 mL of the extract or standard solution. The blank sample was prepared by mixing 2 mL of distilled water with $AlCl_3$. After 10 min, the absorbance was measured at 430 nm. Rutin trihydrate (0–125 mg/L) dissolved in distilled water was used as a standard ($A = 0.0093\gamma + 0.0207$, $r^2 = 0.9979$). The measurements were performed in triplicate for each sample. Results were expressed as mg rutin equivalent per gramme dry weight of orange peel (mg RE/g DW) and calculated as mean ± SD.

2.7. Total Antioxidant Capacity

The total antioxidant capacity of the extracts was determined by the phosphomolybdenum method [20]. An aliquot of 0.3 mL aqueous extract or standard solution was mixed with 3 mL of the reagent solution consisting of 0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate solution, and 4 mmol/L ammonium molybdate. The mixtures were incubated at 95 °C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm and compared with a blank sample. The blank sample was prepared by replacing the sample with an appropriate volume of distilled water. All measurements were performed in triplicate. Ascorbic acid (10–100 mg/L) was used as a standard ($A = 0.0057\gamma + 0.0231$, $r^2 = 0.9966$). Results were expressed as mg ascorbic acid equivalents per gramme dry weight of orange peels (mg AA/g DW) and calculated as mean ± SD.

2.8. DPPH Radical Scavenging Activity

For DPPH radical scavenging activity of orange peel extracts, the method described by Moreira et al. [21] was used with slight modifications. A volume of 250 µL of each sample was mixed with 2000 µL of an ethanolic DPPH solution (0.04 mg/mL). The mixture was left in the dark for 30 min and the absorbance was measured at 517 nm. The calibration curve was prepared using ascorbic acid (10–50 mg/L) instead of Trolox in the original method, and the results were expressed as mg ascorbic acid equivalents per gramme of dry orange peel (mg AAE/g DW). The equation of the standard curve was $A = 0.8583–0.0143\gamma$ ($r^2 = 0.9997$). The blank sample consisted of 96% ethanol. Each extract was analysed in triplicate (mean ± SD).

2.9. Total Carbohydrate Content

The total carbohydrates in the orange peel extracts were determined by the phenol-sulphuric method described by Benvenutti et al. [22]. Briefly, 1 mL of aqueous extract, 3 mL of concentrated sulphuric acid, and 0.8 mL of 5% phenol in water were mixed. The tubes containing the mixtures were incubated at 90 °C for 5 min. After cooling, the absorbance of each sample was measured at 490 nm. The blank sample was prepared by adding distilled water to the reaction mixture instead of the extract. The measurements were carried out in triplicate. Total carbohydrate content was expressed in g glucose equivalent per gramme of dry orange peel (g GE/g DW) using a standard curve for glucose (0.02–0.20 g/L). The equation of the standard curve was $A = 4.0211\gamma + 0.0329$ ($r^2 = 0.9897$). Results were expressed as mean ± SD.

2.10. Pectin Content

The pectin content in the extracts was determined by alcohol precipitation [23]. The pectin was precipitated by adding twice the volume of absolute ethanol and the mixture was allowed to stand overnight at 4 °C. The pectins were separated with a cheesecloth and washed twice with 96% ethanol. They were then dried at 45 °C until constant weight and the pectin yield was calculated using Equation (1):

$$\text{Pectin yield (\%)} = (W_p/W_i) \times 100 \quad (1)$$

where W_p is the weight of dry extracted pectin and W_i is the dry weight of orange peel. All test series were carried out in triplicate.

2.11. Dietary Fibre Content

The total dietary fibre content in the orange peel extracts obtained with subcritical water and in the dry orange peels was determined according to the methods described in AOAC, No. 958.29 [24].

2.12. Phytochemical Screening Assay

Phytochemical screening was carried out to detect different chemical classes in subcritical water extracts of orange peel. The method described by Redhouane et al. [25] was used with slight modifications. The method is based on specific chemical reactions between a particular chemical class and reagents. These screening methods were applied to check the presence of different chemical classes in the extracts.

2.12.1. Free Flavonoids

A few drops of concentrated HCl and a few milligrams of magnesium were added to 1 mL of the extract. The reaction gives an orange-red colour in the presence of free flavonoids.

2.12.2. Anthocyanins

Anthocyanins are identified by adding 10 drops of ammonia to 5 mL of the plant extract. The positive reaction gives a greenish-blue colouration.

2.12.3. Total Tannins

A few drops of ferric chloride solution (5%) were added to 5 mL of the extract. The reaction gives a black or greenish-blue colouration in the presence of tannins.

2.12.4. Gallic Tannins

Sodium acetate (2 g) and a few drops of ferric chloride solution (1%) were added to 5 mL of extract. After stirring, a dark blue colouration appears in the presence of gallic tannins.

2.12.5. Reducing Sugars (Fehling's Test)

Twenty drops of Fehling's reagent were added to 1 mL of extract and 2 mL of distilled water. A positive reaction is marked by the appearance of a brick-red precipitate.

2.12.6. Cardiac Glycosides

A total of 2 mL of the extract were mixed with 2 mL of chloroform and concentrated sulphuric acid was carefully added. The formation of a dark red-brown layer at the interface of the two phases indicated the presence of cardiac glycosides.

2.12.7. Alkaloids

A mixture of 5 mL of the extract, 2 mL of HCl, and 1 mL of Dragendorff reagent gives a red or orange precipitate, indicating the presence of alkaloids.

2.12.8. Coumarins

Five millilitres of the extract and 0.5 mL of ammonia (25%) were mixed. Observation of fluorescence under an ultraviolet lamp at 365 nm indicates the presence of coumarins.

2.12.9. Saponosides

Hydrochloric acid (5 mL, 0.1 mol/L) and 5 mL of NaOH (0.1 mol/L) were introduced separately into two tubes. After the addition of few drops of extract, the tubes were shaken vigorously. The formation of a stable foam indicated the presence of saponosides.

2.12.10. *O*-Heterosides

A total of 5 mL of each extract were mixed with 0.5 mL of HCl and the mixture was heated in a water bath for 15 min. After cooling, 2.5 mL of $CHCl_3$ was added and the resulting organic phase was separated. The appearance of a brown colour after the addition of 0.5 mL of diluted ammonia (50%) indicated the presence of *O*-heterosides.

2.12.11. *C*-Heterosides

A total of 5 mL of distilled water and 0.5 mL of ferric chloride solution (10%) were added to the aqueous phase previously obtained for the *O*-heterosides. The mixture was heated for 30 min and then cooled. The organic phase was separated by mixing with 2.5 mL of dilute NH_4OH (50%). The appearance of a more or less intense red colour indicated the presence of *C*-heterosides.

2.13. Statistical Analysis

At least three independent experiments were performed for each analysis and data were expressed as mean values ± standard deviation (SD). The analysis of one-way variance (ANOVA: Single Factor test) was used to compare the means and determine significant differences ($p < 0.05$). Statistical analysis of the data was done using Microsoft Excel 2013.

3. Results

3.1. SWE Samples

Five different orange peel extracts were prepared at different temperatures and extraction times, with a sample to solvent ratio of 1:20 (*w*/*w*) (Table 1).

Table 1. Orange peel extracts prepared by SWE at different temperatures and extraction times.

Orange Peel Extract	Extraction Temperature (°C)	Extraction Time (min)
Extract 1	200	60
Extract 2	180	60
Extract 3	150	60
Extract 4	150	35
Extract 5	120	5

Extract 1 was obtained in SWE at 200 °C in 60 min, extract 2 at 180 °C in 60 min, extract 3 at 150 °C also in 60 min, and extract 4 was prepared at 150 °C in 35 min. Extract 5 was prepared at 120 °C in a short extraction time of 5 min. All extractions were carried out in a nitrogen atmosphere at a pressure of 15 bar and a frequency of 3 Hz. The ratio of solid to solvent was 1:20 (*w*/*w*) for all extractions.

The prepared extracts were biologically and chemically characterised.

3.2. Total Extraction Yield and Polyphenol Content in Orange Peel Extracts

The total extraction yield, TPC, and TFC are summarised in Table 2.

Table 2. Total extraction yield, TPC, and TFC of the orange peel extracts obtained by SWE.

Orange Peel Extract	Yield (%)	TPC (mg GAE/g)	TFC (mg RE/g)
Extract 1	45.56 ± 0.53^{a}	27.58 ± 0.38^{d}	3.94 ± 0.03^{e}
Extract 2	44.68 ± 0.43^{b}	29.43 ± 0.11^{c}	5.29 ± 0.07^{d}
Extract 3	43.65 ± 0.39^{c}	36.16 ± 0.28^{b}	8.18 ± 0.08^{b}
Extract 4	42.26 ± 0.47^{c}	36.59 ± 0.56^{b}	7.88 ± 0.06^{c}
Extract 5	41.28 ± 0.46^{d}	45.45 ± 0.28^{a}	9.29 ± 0.08^{a}

Yield—extraction yield (g extract/100 g DW); TPC—Total Phenolics Content (in mg GAE/g DW), TFC—Total Flavonoids Content (in mg RE/g DW). The values represent means ($n = 3$) ± SD. Different letters (a, b, c, d, e) in the same column indicate a significant statistical difference in the observed data ($p < 0.05$).

As shown in Table 2, there were significant differences in total extraction yield, TPC, and TFC between all samples, except for extract 3 and extract 4 in yield and TPC values. These samples were extracted at the same temperature (150 °C) but with different extraction durations (60 and 35 min, respectively), indicating that extraction time did not have a significant effect on these parameters. Extract 1, prepared at 200 °C in 60 min, gave the highest yield (45.56%). The TPC gradually increased with a decreasing temperature, with extract 5 giving the highest result (45.45 mg GAE/g DW) under the mildest conditions and with 5 min extraction time. This obviously indicates a very strong thermal degradation of the phenolic compounds from orange peel at temperatures above 120 °C. Similarly, extract 5 obtained the highest TFC result (9.29 mg RE/g DW).

3.3. Antioxidant Capacity and DPPH Radical Scavenging Activity of Orange Peel Extracts

The total antioxidant capacity and DPPH radical scavenging activity of the orange peel extracts obtained by subcritical water extraction are shown in Figure 1.

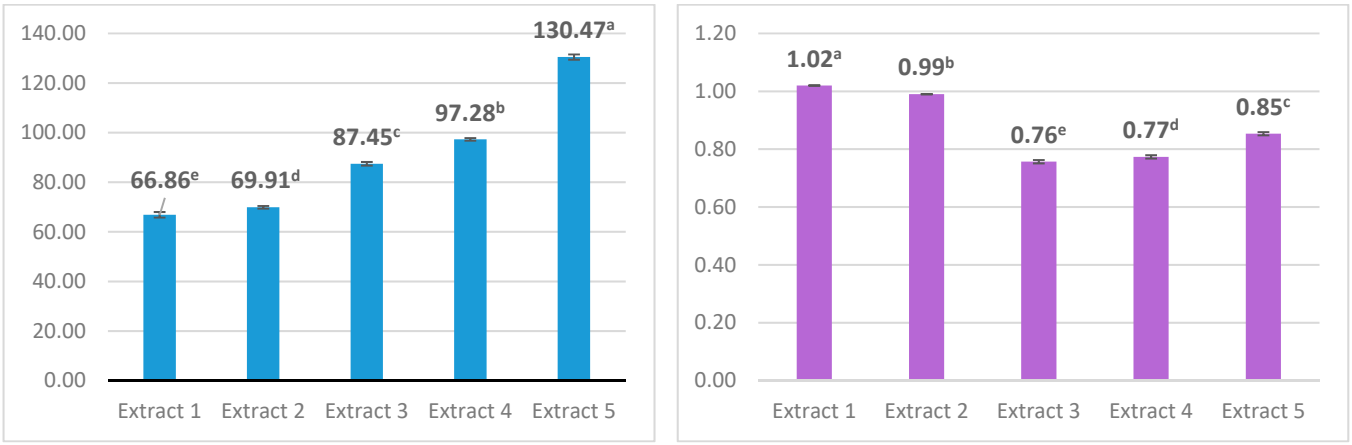

Figure 1. (**a**) Total antioxidant capacity of orange peel extracts (mg AAE/g DW); (**b**) DPPH radical scavenging activity of orange peel extracts (mg AAE/g DW). The error bars indicate standard deviation (n = 3). Different letters (a, b, c, d, e) indicate a significant statistical difference in the observed data ($p < 0.05$).

Figure 1 shows significant differences between the antioxidant and antiradical activities of the extracts obtained at different temperatures and times. Extract 5 gave in the highest total antioxidant capacity (130.47 mg AAE/g DW), which was consistent with the previously determined levels of TPC and TFC in the extracts. The lowest antioxidant activity was observed for extract 1 (66.86 mg AAE/g DW). As for DPPH scavenging activity, the highest value was observed for extract 1 (1.02 mg AAE/g DW), opposing total antioxidant activity, and the lowest for extract 3 (0.76 mg AAE/g DW).

3.4. Carbohydrates in Orange Peel Extracts

The total carbohydrate content (TCC), pectin content, and dietary fibre content (DFC) in the orange peel extracts obtained with SWE are summarised in Table 3.

As shown in Table 3, significant differences were found between the different orange peel extracts. The highest TCC content was obtained for extract 3 (0.48 g GE/g DW) when prepared at 150 °C for 60 min. The lowest TCC content was observed for extract 1 (0.14 g GE/g DW), obtained at 200 °C for 60 min.

Extract 5, obtained at 120 °C for 5 min, had the highest pectin content (23.09%), while the lowest pectin content (5.78%) was observed for extract 1 (200 °C/60 min), indicating its probable degradation.

Dietary fibre content was not detected in extracts 2 and 3, while the content in extracts 1, 4, and 5 ranged from 0.24% to 0.47%. The total dietary fibre content was also determined

directly in the dry orange peel before extraction and was 50.76% (data not shown in the table).

Table 3. Total carbohydrate content (TCC), pectin content, and dietary fibre content (DFC) in orange peel extracts obtained with SWE.

Orange Peel Extract	TCC (g GE/g)	Pectin Content (%)	DFC (%)
Extract 1	0.14 ± 0.01 ^{e}	5.78 ± 0.55 ^{e}	0.47 ^{a}
Extract 2	0.30 ± 0.01 ^{d}	7.24 ± 0.84 ^{d}	n.d.
Extract 3	0.48 ± 0.02 ^{a}	15.05 ± 0.82 ^{c}	n.d.
Extract 4	0.38 ± 0.02 ^{c}	17.06 ± 1.64 ^{b}	0.24 ^{b}
Extract 5	0.43 ± 0.01 ^{b}	23.09 ± 0.90 ^{a}	0.26 ^{b}

TCC—Total Carbohydrate Content (g GE/g DW); Pectin Content (g pectin/100 g DW); DFC—Dietary Fibre Content (%) in orange peel extracts. n.d.—not detected. Values represent means (n = 3) ± SD. Different letters (a, b, c, d, e) indicate a significant statistical difference in the observed data ($p < 0.05$).

3.5. Phytochemical Screening Assay

The chemical classes detected by phytochemical screening of the orange peel extracts produced by SWE are shown in Table 4.

Table 4. Phytochemical screening assay—identified chemical classes in orange peel extracts obtained by SWE.

	Orange Peel Extract				
Chemical Classes	**Extract 1**	**Extract 2**	**Extract 3**	**Extract 4**	**Extract 5**
Free Flavonoids	+	++	+++	+++	+++
Anthocyanins	-	-	-	-	-
Total Tannins	+++	+++	+++	+++	+++
Gallic Tannins	+++	+++	+++	+++	+++
Reducing Sugars	+++	+++	+++	+++	+++
Cardiac Glycosides	++	++	++	++	++
Alkaloids	+	+	++	++	-
Coumarins	+	+	+	+	+
Saponosides	-	-	-	-	-
O-Heterosides	+++	+++	+++	+++	+++
C-Heterosides	+++	+++	+++	+++	+++

(-)—Absence, (+)—presence in low concentrations, (++)—presence in average concentrations, and (+++)—presence in high concentrations.

4. Discussion

Although citrus peels are not edible, they have been shown to be a rich source of a variety of bioactive constituents such as phenolic compounds, vitamins, minerals, terpenoids, terpenes, dietary fibre, and polysaccharides, which are associated with significant biological activities, namely antioxidant, antimicrobial, antidiabetic, and anticarcinogenic activities [26,27]. These compounds also have anti-allergic, anti-ageing, cardioprotective, and neuroprotective properties [12,28,29]. Polyphenols, with flavonoids being the most important class of compounds, are the most abundant bioactive constituents of orange peel, but their nature and content in extracts are influenced by environmental conditions, the variety of the subspecies, and the extraction technique used [12,26].

Further, of the SW extracts obtained in this work, extract 5, prepared at 120 °C for 5 min, achieved the highest TPC (45.45 mg GAE/g DW) as well as the highest TFC (9.29 mg

RE/g DW). The lowest TPC (27.58 mg GAE/g DW) was found in extract 1 (200 °C/60 min), which was almost 40% lower than the TPC observed for extract 5, indicating that thermal degradation occurs at higher extraction temperatures and longer extraction times. In fact, the TPC and TFC of the orange peel extracts increased with decreasing extraction temperatures and times (Table 2), demonstrating the well-known fact that polyphenols (with flavonoids) are heat-sensitive compounds that undergo thermal degradation at high temperatures [30]. Thermal degradation is the most common mechanism used to explain the decline in polyphenol yield in high temperature extractions. Nevertheless, the TPC of the extract prepared at 200 °C for 60 min (27.58 mg GAE/g DW) is not negligible, which can be explained by the bonds between the lignocellulosic materials and the insoluble phenolic acids in the peel. These bonds can be broken and the lignocellulosic material itself can be degraded at high temperatures, producing more phenolic acids, which could explain the relatively high phenolic yield content [31]. Many studies have reported that the extraction temperature has a significant effect on the type of polyphenols which are extracted, as different polyphenols are degraded at different temperatures [31–34]. Besides the quantitative difference in TPC and TFC between all of the extracts, 1–5, they could also differ qualitatively. The phenolics profile should be investigated in a future study, first with thin layer chromatography (TLC) and then with HPLC.

According to the scientific literature, there are large differences between the TPC and TFC values of orange peel extracts obtained by different extraction techniques. Table 5 shows the latest data on the TPC, TFC, and extraction yield of various citrus peel extracts according to different studies.

Table 5. TPC, TFC, and extraction yield of various citrus peel extracts found in previous scientific papers.

Extraction Technique	TPC (mg GAE/g DW *)	TFC	Yield (%)	Reference
Orange peel				
Decoction (water)	9.40	4.20 mg QE/g DW	-	[35]
MAE (aqueous acetone)	12.09	-	-	[36]
UAE (aqueous acetone)	10.35	-		
ASE (aqueous acetone)	6.26	-		
CSE (aqueous acetone)	10.21	-		
Maceration (water)	2.56	0.52 mg CE/g DW	9.40	[26]
Maceration (ethanol)	3.45	0.80	10.90	
Maceration (methanol)	3.24	0.52	15.56	
Maceration (acetone)	3.06	0.58	8.23	
Maceration (petroleum ether)	1.90	0.45	10.16	
Maceration (hexane)	1.53	0.40	11.80	
High-temperature pretreatment; extraction with methanol	22.4	12.7 mg RE/g DW	-	[37]
UAE (aqueous ethanol)	1.86	-	11.00	[38]
NADES (choline chloride-malic acid)	10.53	0.95 mg CE/g DW	-	[39]
SWE	45.45	9.29 mg RE/g DW	41.28–45.56	This work
Lemon peel				
Hydroethanolic extracts	105–204	27–56 mg QE/g	10.64–14.33	[40]
Ethanolic, *n*-hexane, ethylacetate extracts	8.9–15.2	2.49–28.9 mg QE/g	-	[41]

Table 5. *Cont.*

Extraction Technique	TPC (mg GAE/g DW *)	TFC	Yield (%)	Reference
Tangerine peel				
Methanolic extract	122.5	-	-	[42]
Grapefruit peel				
Accelerated solvent extraction	28–85	-	-	[43]
Hexane:methanol:acetone (2:1:1)	10.78	-	-	[44]

* Data are expressed per g dry weight of citrus peel. QE—quercetin equivalent; CE—catechin equivalent. Yield—extraction yield (%).

As reported, SWE was the most efficient technique for use on orange peel in terms of TPC value among all of the extraction methods. The TFC values could not be compared due to the different units used to indicate flavonoid content, although the TFC values obtained in this work were relatively high. Considering all of the results, it can be concluded that SWE is a very high-yielding process for the production of orange peel extracts with a high content of bioactive compounds in extremely short extraction times (5 min) and at relatively low temperatures (120 °C). The extraction yield decreased slightly with a decreasing extraction temperature and was the highest for extract 1 (45.56%). The extraction yield of extract 5 was 41.28%. This value is relatively high considering the mild extraction conditions (120 °C/5 min). Compared to other extraction techniques from the literature, the extraction yield of SWE was several times higher (Table 5).

Together with TPC and TFC, the total antioxidant capacity determined by the phosphomolybdenum method was the highest for extract 5 (130.47 ± 1.06 mg AAE/g DW), while extract 1 had the lowest value (66.86 ± 1.13 mg AAE/g DW), indicating a direct relationship between the polyphenols content and the antioxidant properties of the studied extracts. These results are in line with numerous studies conducted with different plant materials [45–49]. Compared with other results obtained with the same method for different plant extracts, it can be concluded that orange peel extracts obtained by SWE have a very high antioxidant potential. Amo-Mensah et al. [50] investigated the total antioxidant capacity (TAC) of ethanolic extracts from the root and bark of the plant *Vitex grandifolia*, which is widely distributed in Africa. According to the authors, the TAC of the root extracts was 183.9 mg AAE/g DW, with a TPC of 57.2 mg GAE/g DW, while the TAC of the bark extract was 158.7 mg AAE/g DW (with a TPC of 50.9 mg GAE/g DW), which is slightly higher than the results obtained in this study. Umdale et al. [51] reported a TAC of 0.67 mg AAE/g for the methanolic extract of the stems of the Indian plant *Frerea indica*, with a TPC of 4.64 mg GAE/g and a TFC of 6.67 mg RE/g. In the study by Bahadori et al. [52], *Plantago lanceolata* had a TAC of 145 mg AAE/g extract, while da Cruz et al. [53] determined a TAC of 243 mg AAE/g for a hydroethanolic extract of *Alpinia zerumbet*.

In contrast to the total antioxidant capacity, the highest value of DPPH radical scavenging activity was found in extract 1 (1.02 mg AAE/g DW). This phenomenon could be explained by the degradation of thermolabile polyphenols and the formation of new compounds at high extraction temperatures, known as Maillard reaction products, which may have antiradical activity, although some of them are toxic, carcinogenic, and mutagenic [54–56]. As mentioned above, different extraction temperatures may also have influenced the type of polyphenols which were extracted. In general, an increase in extraction temperature (up to 180–200 °C) correlates with an increase in phenolic content and antioxidant activity, but the oxidation process of polyphenols at high temperatures may also lead to a loss of antioxidant activity [31]. In addition, the DPPH reagent may have reacted with other compounds which were present in the extracts, such as carotenoids or vitamin C, which are antioxidant substances that may be present in orange peel [57].

Carbohydrates in plants can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be re-

moved by conventional extraction or washing steps [16]. The total carbohydrates in the studied orange peel extracts were determined by the phenol-sulphuric method [22], which includes all reducing carbohydrates present in the sample (simple sugars, oligosaccharides, polysaccharides, and their derivatives with free or potentially free reducing groups). With decreasing temperature and extraction times, the TCC value regularly increased from 0.14 to 0.43 mg GE/g DW, except for extract 3, whose TCC value was the highest (0.48 g GE/g DW). These results indicate that both reducing carbohydrates and polyphenols can be degraded at higher temperatures, including Maillard reactions between sugars and proteins or by caramelisation reactions. Caramelisation requires temperatures higher than 120 °C and occurs when food surfaces are strongly heated (during baking and frying or wine making), while Maillard reactions can occur at 50 °C and a pH of 4–7 (pH range of the food) [58]. On the other hand, pectin decomposes at temperatures between 150 and 250 °C [10]. Therefore, the processes in extract 3 (prepared at 150 °C during the 60 min) could be the hydrolysis of pectin (and possibly other biopolymers) to mono- and di-saccharides and their further partial degradation by Maillard reactions and caramelisation, which explains it having the highest observed TCC. At higher temperatures (180 and 200 °C), the degradation processes are likely to be more intense during the same extraction time of 60 min, resulting in a lower content of polyphenols and reducing sugars. The TCC content in extract 5 was 0.43 g GE/g DW, which was exceptionally high given the relatively low extraction temperature and time. In contrast to these results, Lachos-Perez et al. [16] observed optimal sugar yields of monomers (arabinose and glucose) at 200 °C in the hydrothermal processing of orange peel biomass, but in flow-through treatments with subcritical water, which differ from the batch treatments performed in this study. The water flow rate in the dynamic processes affects the thermodynamics, mass transfer, and residence time on yield and product degradation [16].

Pectin content increased with decreasing extraction temperatures and times. The highest pectin content was observed in extract 5 (23.09 g/100 g), while the lowest was found in extract 1 (5.78 g/100 g DW). Pectin hydrolysis is expected to occur at high temperatures (200 °C and 180 °C), with D-galacturonic acid, an oxidised form of D-galactose, being the main product. As suggested by Bezus et al. [23], the optimum temperature and time for the SWE of pectin is 120 °C for 5 min.

The soluble fibre content was determined in the orange peel extracts prepared by SWE and in the dried orange peels. The DFC in extracts 1, 4, and 5 was negligible (0.24–0.47%), and no dietary fibre was detected in extracts 2 and 3 (Table 3). However, the dietary fibre content in the dried orange peels was 50.76%, indicating that the solubilisation/hydrolysis of dietary fibre by subcritical water certainly requires very careful temperature adjustment and probably lower temperatures, which is also confirmed by the relatively high carbohydrate contents in all of the extracts (0.14–0.48 g GE/g).

Phytochemical screening confirmed the presence of numerous phytocompounds in the orange peel extracts obtained with subcritical water (Table 4). As mentioned earlier, orange peels are a rich source of flavonoids, of which hesperidin and narirutin are the most abundant [16,27]. Anthocyanins were not detected in the orange peel extracts, but this was to be expected as these compounds are rarely found in citrus fruits, with the exception of blood oranges (*Citrus sinensis* L. Osbeck) [59]. Tannins are a class of astringent polyphenolic biomolecules that bind and precipitate proteins and other organic compounds. Tannins have a molecular weight from 500 to over 3000 Da (gallic acid esters) and up to 20,000 Da (proanthocyanidins and condensed tannins), and form strong complexes with various macromolecules. Condensed tannins are found in practically all plant families [60]. The high content of tannins (total and gallic) was demonstrated in the phytochemical screening of all of the extracts. The presence of cardiac glycosides was also detected, but these results should be further investigated. Phytochemical screening confirmed the presence of alkaloids in the orange peel extracts, with the exception of extract 5. The major alkaloid components in citrus peel are synephrine (about 0.38%) and *N*-methyltyramine. The alkaloid fraction of dried citrus peels has significant antiasthmatic effects, constricts blood vessels, increases blood pressure, and may also improve metabolism and increase calorie

consumption [9]. Coumarin derivatives are produced as secondary metabolites by many plants, bacteria, and fungi. Some of them belong to the class of phenolic compounds. They are used as additives in cosmetic and food industries, but their most important use is in biomedical applications due to their excellent pharmacological and therapeutic properties [61]. The phytochemical investigation conducted in this study revealed that coumarins are present in orange peel extracts in small amounts. *O*-heterosides (*O*-glycosides) are acetals in which a hydroxyl group of a sugar moiety is condensed with a hydroxyl group of a non-sugar moiety. *C*-heterosides (*C*-glycosides) have a sugar moiety linked to a non-sugar moiety (aglycone) via a carbon–carbon bond. Phenolic heterosides are widely distributed in the plant kingdom [62]. Both *O*- and *C*-heterosides are detected in high concentrations in orange peel extracts (Table 4).

All the results obtained in this study are clear evidence of the richness of SWE-derived orange peel extracts in health-promoting compounds. Future applications of the presented results should be directed towards the study of the chemical composition of the extracts obtained at different temperatures, which have different properties, using chromatography methods. Depending on the phenolic profile and after appropriate clinical studies, these extracts could find their potential use in medicine and cosmetics, and as food additives.

5. Conclusions

Orange peel extracts obtained with subcritical water at different temperatures (120–200 °C) and extraction times (5–60 min) were found to be rich sources of bioactive compounds, namely polyphenols (including flavonoids), pectin, and carbohydrates, and have high antioxidant (antiradical) activity. Of all the chemically characterised orange peel extracts, the one obtained at a relatively low temperature of 120 °C and an extremely short extraction time of 5 min had the highest TPC (45.45 mg GAE/g DW), TFC (9.29 mg RE/g DW), total antioxidant capacity (130.47 mg AAE/g DW), and pectin content (23.09%). The same extract achieved one of the highest total carbohydrate contents (0.43 g GE/g DW). Phytochemical screening confirmed the presence of free flavonoids, tannins, reducing sugars, alkaloids, coumarins, and heterosides in the orange peel extracts. All of our results suggest that SWE is a powerful tool that could be a promising source of health-promoting compounds from orange peel. After further clinical studies, the orange peel extracts obtained by subcritical water have the potential to be used in newer medicinal preparations, cosmetics, and functional foods.

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