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Polyphenol Extraction by Different Techniques for Valorisation of Non-Compliant Portuguese Sweet Cherries towards a Novel Antioxidant Extract

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Abstract: Currently, there is special interest in the recovery of polyphenols from non-compliant fruits that have no market value; efforts to find value-added solutions for these food areas are a key option for a sustainable bio-economy. Saco cherries are a traditional Portuguese cherry variety, and although they are a nutritionally important food, rich in powerful dietary polyphenols, significant amounts of these cherries are not sold due to their small size. In this context, this work aimed to select the best method to produce novel antioxidant polyphenol-rich extracts from low calibre and non-compliant Saco cherries. Based on the results, microwaves-assisted extraction (MAE) allowed us to obtain a polyphenol-rich extract with a high antioxidant capacity (50.46 ± 1.58 mg Trolox equivalent (TE)/g dry extract (DE) by oxygen radical absorbance capacity (ORAC), 10.88 ± 0.38 mg ascorbic acid equivalent (AA)/g DE by 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and 9.58 ± 0.42 mg TE/g DE by 2,2-diphenyl-1-picrylhydrazyl (DPPH)) and a high content of polyphenols, namely, hydroxycinnamic acids (neochlorogenic and *p*-coumaric acids) and anthocyanins (cyanidin-3-rutinoside and cyanidin-3-glucoside), compared with those of conventional extractions with low and high temperature and ultrasound-assisted extraction. The antioxidant extract produced from MAE could be a new alternative for the valorisation of non-compliant cherries since these extracts proved to be a functional ingredient due to the high content of antioxidants, which are linked to the prevention of diseases.

Keywords: Saco cherry; non-compliant; polyphenols; antioxidant activity; extraction method

1. Introduction

Sweet cherries (*Prunus avium* L.) are among the most attractive fruit to consumers, due to their taste, sweetness, firmness, colour [1], and a high content of health-promoting compounds [2]. Beira Interior is a Portuguese region that has the largest sweet cherry production in Portugal with the protected geographical indication (PGI) registered Cova da Beira cherry. Annually, the production of Cova da Beira cherries reaches around 17,000 t, with the Saco sweet cherry variety being the oldest and the most produced [3,4]. In the context of a bio-economy, the Saco cherry variety is the most important in the Cova da Beira region. Climate change, phytosanitary uncertainties, and the dependence of consumers on fresh fruit have led to the need for research and implementation of new high-value pathways for low-calibre and non-compliant fruit, without compromising sustainability, by increasing the circular

bio-economy in this sector (decreasing the food losses). Several studies showed that Saco cherries have interesting characteristics from nutritional and bioactive points of view, mainly associated to their composition of different dietary phenolic compounds such as phenolic acids, flavonoids, anthocyanins, flavan-3-ols, and flavanols; however, the anthocyanins are the most interesting polyphenols present in sweet cherries [4,5]. Furthermore, these phenolic compounds are correlated with great antioxidant activity, which is associated with improved health benefits, playing an important role in preventing several chronic diseases [5]. Saco cherry production generates a high amount of non-compliant fruit, i.e., fruit that does not meet the standards required to be marketed due to its low calibre/size. Furthermore, sweet cherries are highly perishable fruits with a short shelf-life and postharvest-life, and are expensive to acquire and produce [6]. However, the traditional varieties are a source of distinctive genetic characters derived from many years of adaptation to the original territory [7] and reflect the high Portuguese biodiversity. Due to the aforementioned constraints due to non-compliant fruit, Saco cherries do not have high value in the market and, for that reason, are considered a food loss, which if not reused, will lead to negative environmental and economic impacts [8]. This represents an opportunity in the agricultural sector in terms of biodiversity preservation, environmental sustainability, and valorisation of the final products [7].

In the past few years, there has been an increased interest in the recovery of bioactive compounds (BCs) from this kind of fruit to comply with the circular economy concept. Similarly, social awareness linked to natural food markets has been rising and focusing on food losses valorisation and a move towards natural ingredients free of harmful chemicals to preserve personal health and safety [9]. However, when producing extracts, there is the need to keep the integrity of the active molecules and assure, at the same time, consumer safety and environmental sustainability.

The development of new strategies to meet new market demands when producing natural antioxidant extracts is of extreme importance because the commonly used conventional extraction (CE) methods, such as maceration, consume high amounts of solvents, time, and energy, and are a hazard to the environment [10]. Green chemistry-based extraction methods could be the option applied to meet the circular economy goals, reducing the negative environmental impact of the conventional extraction methods. Hence, the optimization of these processes via the development of an appropriate non-toxic extraction solvent system or via the use of emergent extraction techniques has been studied [11]. Theoretically, the optimal extraction method should be simple, safe, reproducible, inexpensive, and suitable for industrial application [12]. As CE usually takes more time and requires large volumes of solvents, nonconventional extraction methods such as microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), among others, are being used for the recovery of polyphenols and other compounds from fruits like cherries [13,14]. Currently, great attention has been given to green extraction technologies, since they can reduce or eliminate the use of hazardous substances and limit the cost of solvent waste disposal [15]. MAE allows for high-temperature extraction, but with reduced extraction times, thereby consuming less energy. The amount of solvent used is smaller when compared to that used by conventional extraction methods. On the other hand, UAE has low extraction time with high recovery yields, but in contrast to MAE, this extraction applies lower temperatures. UAE has been widely applied to the production of bioactive extracts from plants and fruits since it is especially good at breaking the cell walls, leading to the release and recovery of high-quality BCs [13,16]. So far, three research works studied the production of polyphenolic-rich extracts from Saco cherries using conventional hydro-alcoholic extraction without high temperature, followed by separation and adsorption processes [8,17], and through two-steps of supercritical CO_2 extraction [18]. To the best of our knowledge, this is the first time that MAE and UAE were applied to non-compliant Saco cherries to produce novel antioxidant extracts.

In this work, two green extraction techniques (MAE and UAE) were compared with CE (with low and high temperatures) to select the best technique to produce a novel antioxidant polyphenol-rich extract from non-compliant Portuguese Saco sweet cherries for further applications as a functional ingredient with antioxidant activity in food products.

2. Materials and Methods

2.1. Chemicals

The 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-caffeoylquinic acid, 4-caffeoylquinic acid, 4,5-Di-O-caffeoylqinic acid, 5-caffeoylquinic acid, ascorbic acid, caffeic acid, fluorescein, gallic acid, *p*-coumaric acid, potassium chloride (KCl), sodium acetate (CH_3CONa), sodium carbonate (Na_2CO_3), trifluoroacetic acid (TFA), and trolox were purchased from Sigma-Aldrich (Sintra, Portugal). (−)-Epicatechin-3-gallate, cyanidin-3-glucoside, cyanidin-3-rutinoside, kaempferol-3-glucoside, pelargonidin-3-rutinoside, peonidin-3-rutinoside, quercetin, and quercetin-3-rutinoside were purchased from Extrasynthése (Genay Cedex, France). Acetonitrile and methanol were purchased from Fischer Scientific (Oeiras, Portugal). Folin-Ciocalteu's reagent, hydrochloric acid 37 % (*v*/*v*) (HCl), and potassium persulfate were purchased from Merck (Algés, Portugal).

2.2. Fruit Material and Preparation of Extracts

2.2.1. Fruit Material

Low calibre and/or non-compliant sweet cherries (*Prunus avium* L.) from the Saco variety, grown in Fundão-Cova da Beira, Portugal (40°08′24.90′′ N-7°30′4.86′′ W), were provided by "Cerfundão-Embalamento e Comercialização de Cereja Cova da Beira, Lda." company from Fundão, in May 2018 at the commercial maturity stage. The cherries were stored in a cold room at 6 °C during the analysis time (2 weeks). The stems and the pits were removed by hand and washed in cold water, and then dipped in 100 µg/L of sodium hypochlorite solution for 1 min. After that, the cherries were crushed to obtain a homogenous pulp, before the extraction procedures.

2.2.2. Preparation of Saco Cherry Extracts

Classical extraction with low temperature (CE) and with high temperature (CET): The extraction with low temperature was made at room temperature (23 °C) over 1 h by stirring 10 g of crushed cherries with 30 mL ethanol/water (50:50, *v*/*v*) acidified with 0.1% of HCl, previously homogenized using Ultra Turrax (Ultra Turrax T-25, IKA, Germany) at 9500 rpm for 60 s. Extraction with high temperature was performed in the same way, but after homogenization, extraction continued with stirring at 65 ± 1 °C over 30 min.

Microwaves-assisted extraction (MAE): 10 g of crushed cherries were homogenized with 30 mL ethanol/water (50:50, *v*/*v*) acidified with 0.1% de HCl using an Ultra Turrax at 9500 rpm for 60 s. The extraction was made using a single-mode focused microwave reactor (Milestone, Start S Microwave Labstation for Synthesis, Italy, with a rotor SK-12) operating at 2450 MHz with adjustable microwave power, without exceeding 65 °C. General extraction parameters were: 300 W during 15 min, divided into 3 identical cycles of 5 min, with an interval of 5 min between cycles.

Ultrasound-assisted extraction (UAE): 10 g of crushed cherries were homogenized by Ultra Turrax at 9500 rpm for 60 s with 30 mL ethanol/water (50:50, *v*/*v*) acidified with 0.1% of HCl. The sonication proceeded for 30 min in an ultrasonic bath (Laborette 17, Fritsch, Germany) with 120 W application power and a frequency range of 50–60 Hz with temperature control (30 °C).

Afterwards, all the extracts obtained above were cooled at room temperature and were centrifuged (K241, Centurion Scientific Ltd., Chichester, UK) at 2600 G for 20 min. The supernatant was retained, the ethanol fraction evaporated at 40 °C, 175 mbar pressure by rotary evaporator, and the remaining aqueous extract was reduced to powder by freeze-drying (LyoQuest-85, Telstar, Portugal). All extraction

techniques were done in three independent extractions. The extraction yield was calculated based on the amount of fresh Saco cherry used to make the extracts (Equation (1)).

$$\text{Extractive Yield } (\%) = \frac{\text{Dried extract } (g)}{\text{Fresh weight of fruit } (g)} \times 100 \quad (1)$$

2.3. Total Phenolic Content

Total phenolic content (TPC) of the cherry extracts was determined by Folin–Ciocalteu method [19] with some modifications. Briefly, 80 µL of Folin–Ciocalteu reagent 10% (*v*/*v*) was added to 20 µL of extract (previously dissolved in distilled water), followed by 100 µL of sodium carbonate (7.5% (m/v)) and allowed to react in the dark at room temperature (23 °C) for 1 h. After that, absorbance was measured at 750 nm (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA) in a 96-well microplate (Nunc™, Thermo Fisher Scientific Inc., Waltham, MA, USA). Gallic acid was used as a standard for the calibration curve (0.010–0.125 mg/mL, $y = 5.991x + 0.126$, $R^2 = 0.999$) and the results were expressed as milligrams equivalent of gallic acid per gram of dry extract (mg GAE/g DE). Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

2.4. Total Anthocyanins

Total anthocyanins content (TAC) of cherry extracts was determined by the pH-differential spectroscopic method described by Lee et al. [20]. Briefly, 250 µL of each extract (previously dissolved and after diluted in distilled water) was mixed with 750 µL of two different buffers: Potassium chloride (KCl) at pH 1.0 and sodium acetate (CH_3CONa) at pH 4.5. The absorbance was measured at 515 and 700 nm (UV mini 1240, Shimadzu, Tokyo, Japan) after incubation for 20 min at room temperature (23 °C). Absorbance (A) was calculated as follows in Equation (2), and then the TAC, in mg/L, was estimated by Equation (3). Finally, the results were converted and expressed as milligrams of cyanidin-3-glucoside equivalent per g of dry extract (mg Cy-3-glu/g DE).

$$A = (\text{Abs}_{515\,nm} - \text{Abs}_{700\,nm})_{pH1.0-} (\text{Abs}_{515\,nm} - \text{Abs}_{700\,nm})_{pH4.5} \quad (2)$$

$$C\ (mg/L) = (A \times MW \times DF \times 1000) \div \varepsilon \times L \quad (3)$$

where molecular weight (MW) and molar extinction coefficient (ε) of cyanidin-3-glucoside is 449.2 g/mol and L/mol*cm, respectively; cuvette optical path length (L) is 1 cm, and final dilution factor (DF) is 80. Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

2.5. Phenolic Compounds Identification by LC-ESI-QqTOF-HRMS

Antioxidant extracts were dissolved in ultrapure water at 20 mg/mL for further analysis by LC-ESI-UHR-QqTOF-MS according to Monforte et al. [21] with some modifications. The separation was performed in a UHPLC UltiMate 3000 Dionex (Thermo Scientific), coupled to an ultrahigh-resolution, Qq-time-of-flight (UHR-QqTOF) mass spectrometer with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany). Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100 mm × 2.1 mm, 2.2 µm) (Dionex). Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Separation was carried out over 24.5 min under the following gradient conditions: 0 min, 0% B; 10 min, 21.0% B; 14 min, 27% B; 18.30 min, 58%; 20.0 min, 100%; 24.0 min, 100%; 24.10 min, 0%; 26.0 min, 0% at a flow rate of 0.25 mL/min. The injection volume was 5 µL. Parameters for MS analysis were set using negative ionization mode with spectra acquired over a range from m/z 20 to 1000. The parameters were as follows: Capillary voltage, 3.0 kV; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision RF, 300 Vpp; transfer time, 120 µs; and prepulse storage, 4 µs.

Post-acquisition internal mass calibration used sodium formate clusters, with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis. High-resolution mass spectrometry was used to identify the compounds. The elemental composition for the compound was confirmed according to accurate mass and isotope rate calculations designated mSigma (Bruker Daltonics). The accurate mass measurement was within 5 mDa of the assigned elemental composition, and mSigma values of <20 provided confirmation. Compounds were identified based on its accurate mass $[M\text{-}H]^-$. Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

2.6. Phenolic Compounds Quantification by HPLC

The quantitative profile of phenolic compounds in cherry extracts (previously dissolved in ultrapure water) was performed using a Waters Alliance e2695 separation module system interfaced with a photodiode array UV/Vis detector 2998 (PDA 190–600 nm) (Waters, Mildford, MA, USA). The separation of the compounds was carried out in a reverse-phase C18 column (COSMOSIL 5C1 8-AR-II Packed Column–4.6 mm I.D. × 250 mm; Dartford, UK). The mobile phases and the gradient program used were prepared according to Oliveira et al. [22] with some modifications. The mobile phase was composed of solvent A: Water/acetonitrile/TFA (94.9/5/0.1%) and solvent B: Acetonitrile/TFA (99.9/0.1%) with the elution gradient: 0–1 min 0% B; 1–30 min 21% B; 30–42 min 27% B; 45–55 min 58% B; 55–60 min 0% B, and kept another 1 min at 0% B. Flow rate was 1 mL/min, the oven temperature was set as 25 °C, and the injection volume was 20 µL. Detection was performed at 280 nm, 320 nm, 360 nm, and 520 nm, while data acquisition and analysis were accomplished using Software Empower 3. Identification of compounds was done by comparing the retention times and spectra with pure standards (3-, 4-, 5-, 4,5-caffeoylquinic acids, caffeic acid, epicatechin-3-gallate, kaempferol-3-glucoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-rutinoside, pelargonidin-3-rutinoside, protocatechuic acid and *p*-coumaric acid, quercetin-3-rutinoside, and quercetin). Herein, *p*-coumaroylquinic acid was calculated as equivalents of *p*-coumaric acid. The quantification was performed by the calibration curves and the results were expressed as milligrams per gram of dry extract (mg/g DE). In Table 1 are present the validation criteria of the phenolic compounds for the equipment. Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

Table 1. Calibration curves, Limit of Detection (LoD), and Limit ofQuantification (LoQ) of pure standards use for quantification of polyphenols by HPLC, and results for the repeatability and recovery.

Phenolic Compounds	Validation Criteria						
	Slope	Intercept	R^2	LoD (mg/mL)	LoQ (mg/mL)	Repeatability (% RSD)	% Recovery
Hydroxycinnamic acids							
3-Caffeoylquinic acid	54,993,453	195,577	0.998	0.02	0.06	0.382	92.684
5-Caffeoylquinic acid	57,646,292	302,485	0.999	0.01	0.04	0.838	101.219
4-Caffeoylquinic acid	55,692,713	116,655	0.999	0.01	0.05	0.430	98.995
4,5-dicaffeoylquinic acid	31,929,586	91,582	0.999	0.01	0.03	0.566	97.548
Caffeic acid	109,109,858	485,096	0.998	0.01	0.06	0.139	99.510
***p*-coumaric acid**	110,590,209	213,534	0.999	0.01	0.04	0.331	99.801
Flavan-3-ols							
(−)-Epicatechin-3-gallate	35,497,526	38,356	0.999	0.01	0.02	0.417	98.887
Flavonols							
Quercetin-3-rutinoside	31,584,226	132,541	0.998	0.02	0.05	0.162	98.591
Kaempferol-3-glucoside	40,663,899	30,580	0.999	0.01	0.04	1.087	95.975
Quercetin	86,817,569	360,940	0.998	0.02	0.06	0.210	96.823
Anthocyanins							
Cyanidin-3-rutinoside	53,088,951	91,623	0.999	0.01	0.02	0.855	100.749
Cyanidin-3-glucoside	13,535,732	−39,433	0.999	0.01	0.03	0.346	97.235
Peonidin-3-rutinoside	51,163,043	256,007	0.998	0.02	0.06	0.411	98.820
Pelargonidin-3-rutinoside	46,960,315	−54,554	0.999	0.01	0.04	0.471	100.005

2.7. Antioxidant Activity

The antioxidant activity of cherry extracts was determined using three different methods:

2.7.1. The ABTS Method

Was performed according to Gião et al. [23]. Briefly, the free radical ABTS° was generated through a chemical oxidation reaction with potassium persulfate. After that, the concentration of ABTS° was adjusted with water to an initial absorbance of 0.700 ± 0.020 at 734 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sample (15 µL), previously dissolved in distilled water, was allowed to react in the dark at room temperature (23 °C) with 200 µL of diluted ABTS° solution, and the absorbance was read exactly 6 min after initial mixing in a 96-well microplate (Nunc™, Thermo Fisher Scientific Inc., Waltham, MA, USA). A blank was taken with distilled water (A0). The inhibition percentage (I) of the sample was calculated using Equation (4) and compared with the ascorbic acid standard calibration curve (0.0088–0.088 mg/mL, $y = 1103.3x - 6.206$, $R^2 = 0.999$). The results were expressed as milligrams of ascorbic acid equivalent per gram of dry extract (mg AAE/g DE). Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

2.7.2. The DPPH Assay

Was carried out according to the procedure described by Alexandre, Silva, Santos, Silvestre, Duarte, Saraiva, and Pintado [19], with some modifications. Briefly, a stock solution (600 µM) was prepared by dissolving 24 mg of DPPH in 100 mL of methanol, and stored at −20 °C in the darkness. Work solution (60 µM) was prepared to mix 10 mL of stock solution with 90 mL of methanol so absorbance reached 0.700 ± 0.02 at 515 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sample (25 µL), previously dissolved in distilled water, was allowed to react with the DPPH° work solution (175 µL) in the dark at room temperature (23 °C) for 30 min in a 96-well microplate (Nunc™, Thermo Fisher Scientific Inc., USA). Then, the absorbance was measured at 515 nm and a blank was taken with distilled water (A0). The inhibition percentage (I) of the sample was calculated using Equation (4), and Trolox was used as a standard to prepare a calibration curve (0.0075–0.075 mg/mL, $y = 1176.3x - 1.093$, $R^2 = 0.999$). The results were expressed as milligrams of Trolox equivalent per gram of dry extract (mg TE/g DE). Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

$$I\ (\%) = [(Abs_{\ A0} - Abs_{\ sample}) \div Abs_{\ A0}] \times 100 \quad (4)$$

where $Abs_{\ A0}$ is the absorbance of blank, and $Abs_{\ sample}$ is the absorbance of the reaction between the sample and the radicals.

2.7.3. The Oxygen Radical Absorbance Capacity Assay (ORAC)

Was performed in a black 96-well microplate (Nunc, Denmark), following the method described by Dávalos et al. [24] with some modifications. The reaction was carried out in a 75 mM phosphate buffer (pH 7.4), with a final reaction mixture of 200 µL. The sample (20 µL), previously dissolved in distilled water and fluorescein (120 µL; 70 nM, final concentration in well) solution, was placed in the well of the microplate. A blank (FL + AAPH) using phosphate buffer instead of the antioxidant solution (Trolox) and eight calibration solutions ((1–8 µM of Trolox, final concentration in well), $y = 1.39 \times 10^6x + 1.61 \times 10^7$, $R^2 = 0.995$) as antioxidant was also carried out in each assay. The mixture was preincubated for 10 min at 37 °C. AAPH solution (60 µL; 12 mM, final concentration in well) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader, and the fluorescence recorded at intervals of 1 min over a period of 80 min. A multidetector plate reader (Synergy H1, Biotek, Winooski, Vermont, USA) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the Gen5 Biotek software version 3.04. AAPH and Trolox

solutions were prepared daily, and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor fluorescence blank, $t = 0$/fluorescence control, $t = 0$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated according to the trapezoidal method (Equation (5)):

$$AUC = \sum_{i=1}^{i=n-1} \left(\frac{f_i + f_{i+1}}{2} \right) * (t_{i+1} - t_i) \quad (5)$$

where f_i is the fluorescence at reading i, and t_i is the time (minutes) at reading i.

The final AUC values were calculated by subtracting the AUC of the blank from all the results. Final ORAC-FL values were obtained by interpolating in the standard curve, and were expressed as mg of Trolox equivalents/g of dry extract (mg TE/g DE). Three independent analyses were performed in each of the triplicate extracts obtained for each different methodology.

2.8. Statistical Analysis

Results were presented as the average ± standard deviation of three independent extractions ($n = 3$). The normality of data distribution was tested by Shapiro–Wilk test, the homogeneity of variances by Levene's test, and the significance of the differences between extracts was tested by the one-way analysis of variance (ANOVA). The null hypothesis that all means are equal was rejected when the difference between means was $p < 0.05$. Following the ANOVA, tests of multiple comparisons were done at those statistically significant variables using the Tukey's post-hoc test (homogeneity of variance was assumed) at the $p < 0.05$ significance level. All this statistical analysis was performed using SPSS version 23. Additionally, the principal component analysis (PCA) using Statgraphics Centurion XVII software was performed on the data set after normalization, and factor analysis was performed in order to reduce and explain the variability of the data. The Varimax method was used to produce orthogonal transformations to the reduced factors as to better identify the high and low correlations.

3. Results and Discussion

Four different antioxidant polyphenolic-rich extracts were prepared from low-calibre and non-compliant Saco variety cherries, aiming to choose the best methodology for developing added-value ingredients with antioxidant activity, since, commonly, these non-commercial traditional varieties contain more BCs than other exotic varieties that have more production yield [4,25]. The different hydro-alcoholic antioxidant extracts were further evaluated in terms of phenolic compounds composition and antioxidant capacity using chemical in vitro assays.

3.1. Extractive Yield

The main concern of extraction was to obtain the BCs of interest and to maximize the yields of extraction, while minimizing the concentration of undesirable compounds, such as sugars and proteins, that could interfere on the extract stability and quality. Several factors may affect the extraction efficiency being the most important: Solvent, the ratio between mass and solvent, as well as temperature and pH. Since one of the main purposes of this work was to produce extracts, by the application of four different methodologies, following the evaluation of their bioactivities, the variables regarding type of solvent and ratio were fixed. For that, a mixture of water:ethanol (50:50, *v*/*v*) was selected, because (i) these are food grade solvents, and thus are considered to be safe for the consumer even if not completely removed from the final ingredient, (ii) are environmentally friendly, (iii) could be reused in the process, and finally, (iv) this mixture is one of the most common mixtures of solvents used to produce extracts rich in BCs [17]. The extraction yield of the different Saco cherry antioxidant polyphenol-rich extracts obtained by different extraction techniques are shown in Figure 1. The MAE allowed obtaining higher extractive yields (17.03 ± 0.65% *w*/*w*), while the remaining extraction techniques recovery yield was between 12–13% *w*/*w*

($p > 0.05$). It is important to highlight that the higher extractive yield does not always mean extraction of a higher content of phenolic compounds, since some conditions may promote the extraction of other fruit matrix components solubilized according to the extraction methodology. Since the extracting solvent, the ratio, and moisture content in the fruit were the same in all extraction techniques, it can be concluded that microwave effects were responsible for the highest extractive yields (17.03 ± 0.65%), while the mass transfer limitation in CE, CET, and UAE caused the lowest yields (13.41 ± 0.48, 12.66 ± 0.79, and 12.63 ± 0.92%, respectively). Higher yields on MAE, when compared with the conventional heat reflux method (CET), could be due to the homogenous heating of the microwave irradiation mechanism, which results in the temperature increasing from inside the system to the outside, leading to cell disruption and consequent phenolic compounds migration to the solvent [26]. In contrast, CET increased the temperature from outside to inside, and is not so efficient [27]. Temperature influences the extraction efficiency, since higher temperature promotes an increase of the solute solubility in the solvent, increasing the solute rate diffusion into the solvent bulk, leading to a higher mass transfer rate [28]. However, the use of high temperature in conventional extraction (CET) in this study did not increase the extractive yield when compared to conventional extraction with low temperature (CE) (from 25 to 65 °C). Several works revealed that the use of temperature associated with a conventional extraction helps to access a better extractive yield and, consequently, higher polyphenols content [29]. However, the effect of temperature cannot be generalized since it strongly depends on the type of compounds extracted. As reported by other authors [30,31] the UAE was developed to improve the recovery of nutraceuticals in comparison to conventional solvent extraction methods, and there are several studies in phenolic compounds extraction using UAE that showed better extractive yields than conventional extraction methods [32–34]. However, in this work the UAE extractive yields were not statistically different ($p > 0.05$) from the conventional extraction (CE and CET). Extraction with ultrasonic power occurs due to cell disruption, which means that the mass transfer is intensified due to solvent penetration into plant material [35]. So, the UAE may work as a pre-treatment that helps the cells' membrane disruption, while the major benefit of microwaves is the homogenous, rapid, and efficient heating of the system, which results in the expansion and rupture of cell walls, increasing solvent penetration. Similar results to this study were observed in cherry laurel extracts by Karabegović, Stojičević, Veličković, Todorović, Nikolić, and Lazić [26], who reported that MAE had highest extraction yields, followed by UAE and conventional extraction (65 °C during 15 min). However, this study showed lower yield extractions when compared with our results, and this might be due to lower extraction time, lower potency and frequency used, as well as the different extraction solvent (methanol). The lowest extractive yield obtained by CE was also expected, because MAE and UAE were created in order to improve the recovery of nutraceuticals in comparison to conventional solvent extraction methods [36]. The enhancement of extraction with ultrasonic power is due to the intensification of mass transfer and solvent penetration into plant material, as well as cell disruption [35], while a major benefit from microwaves is the rapid, efficient, and homogeneous heating of the total extraction system volume which also results in the expansion and rupture of cell walls and increased solvent penetration [37].

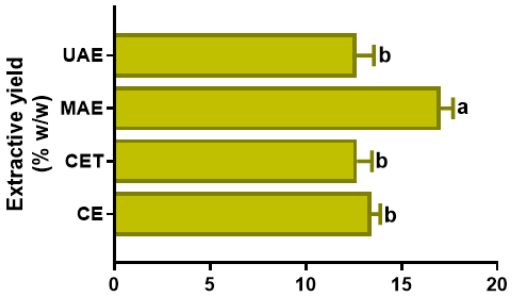

Figure 1. Extractive yield (% *w*/*w*) obtained by different extraction techniques from non-compliant Saco cherry. Different letters mean significant differences between extraction techniques, determined by Tuckey's test ($p < 0.05$).

3.2. Total Phenolic Content and Total Anthocyanins

Phenolic compounds are widely distributed in the plant kingdom, such as in cherries, which are widely known for their biological activities. These compounds offer potential health benefits, especially due to the antioxidant activity [38]. The antioxidant extracts were characterized in terms of total phenolic content (TPC) and for total anthocyanins content (TAC). Table 2 shows the results of the TPC, varied between 8.75 ± 0.81 and 12.65 ± 0.81 mg GAE/g of DE, and the TAC, varied between 1.76 ± 0.06 and 2.78 ± 0.18 mg Cy-3-glu/g DE. The extracts with the highest TPC were obtained in MAE, followed by CE > UAE > CET, without significant differences between UAE and CET ($p > 0.05$). The MAE was shown to be able to generate extracts with higher values of TPC, while CE showed the lowest values. Likewise, many researchers have confirmed the MAE efficiency for phenolic compounds extraction compared to traditional extraction techniques [26,39]. Since it was already mentioned that the higher yields obtained with MAE can be related to the use of more homogeneous heating [26]. However, thanks to this effect, this technology provides a higher yield of other compounds present in the matrix, and not only a selective extraction of polyphenols [40]. Anthocyanins are present in sweet cherry and are attributed to its higher antioxidant activity compared to other classes of phenolic compounds [41]. The anthocyanins are unstable in neutral or alkaline conditions, so the addition of lower acid quantity was beneficial for the anthocyanins extraction, but can negatively influence other phenolic groups, such as chlorogenic acids and flavanols [42,43]. The CET and MAE extracts showed higher values of TAC 2.78 ± 0.18 and 2.53 ± 0.13 mg Cy-3-glu/g DE ($p > 0.05$), respectively. Meanwhile, the CE (1.93 ± 0.21 mg Cy-3-glu/g DE) and UAE (1.76 ± 0.06 mg Cy-3-glu/g DE) showed the lower TAC ($p > 0.05$). Nevertheless, the heating created in MAE compared to conventional heating (CET), MAE had several advantages, like higher heating rate and purity of the final product, shorter extraction time, and lower power energy needed to run the extraction process [40].

Table 2. The Total Phenolic (TPC) and Total Anthocyanins (TAC) Content in Saco cherry extracts. Values are represented by the average ± standard deviation. Different letters mean significant differences between extraction techniques, determined by Tukey's test ($p < 0.05$). CE: Conventional extraction; CET: Conventional extraction with high temperature; MAE: Microwaves-assisted extraction; UAE: Ultrasound-assisted extraction.

Saco Cherry Extracts	TPC (mg GAE/g DE)	TAC (mg Cy-3-glu/g DE)
CE	8.75 ± 0.81 ^{b}	1.93 ± 0.21 ^{b}
CET	7.11 ± 0.33 ^{c}	2.78 ± 0.18 ^{a}
MAE	12.65 ± 0.81 ^{a}	2.53 ± 0.13 ^{a}
UAE	7.16 ± 0.81 ^{c}	1.76 ± 0.06 ^{b}

The TPC and TAC of fruit extracts reflected the extraction yields, where MAE promoted higher content of phenolics than the other methods. However, phenolics only represented 1.26% of the total SC cherry extract. This result can be related to other compounds simultaneously extracted, which account for the total extract mass. Compounds like sugars, polysaccharides, minerals, organic acids, soluble and insoluble fibres (lignin, cellulose, hemicellulose, etc.) are also extracted by the different extraction methods. Folin–Ciocalteu reagent lacking specificity can also interact with the aforementioned molecules, since it is a reaction based on electron-transfer, reducing not only polyphenols, but also sugars and proteins [44], which at the end can lead to some interference in the final measurements [45,46]. Moreover, this method is a total quantification, so it only provides an estimate of total polyphenols content present, therefore a detailed analysis of individual phenolic compounds was performed by LC-ESI-QqTOF-HRMS, and the most abundant were quantified by HPLC-DAD.

3.3. Phenolic Compounds Profile of Antioxidant Extracts

3.3.1. Identification of Major Phenolic Compounds by LC-ESI-QqTOF-HRMS

The analysis by LC-ESI-QqTOF-HRMS allowed a full characterization of the phenolic compounds and the identification of 5 chemical classes of phenolic compounds in Saco cherry extracts, including hydroxycinnamic acids, hydroxybenzoic acids, flavanols, flavonols, and anthocyanins. Thus, around 40 phenolic compounds were identified respectively on Saco cherry extracts (Table 3). The main phenolic acids identified were hydroxycinnamic acids, of which chlorogenic acids (CGAs), were widely distributed in Saco cherry. The 3 main compounds belonging to CGAs were 3-caffeoylquinic, 4-caffeoylquinic, and 5-caffeoylquinic acids. They had a molecular ion $[M-H]^-$ at m/z 353, which was consistent with the molecular formula of $C_{16}H_{18}O_9$. This group of compounds was mainly found in the cherry exotic varieties, as reported by previous studies [1,5], and in Portuguese varieties, as reported by Gonçalves et al. [47] and Serra, Duarte, Bronze, and Duarte [4]. However, Matias, Rosado-Ramos, Nunes, Figueira, Serra, Bronze, Santos, and Duarte [8] only detected 3-caffeoylquinic (neochlorogenic acid) by LC-MS/MS analysis in a (Poly)phenol-Rich Extract obtained by CE with EtOH:H2O (1:1 *v*/*v*), for 2 h, under continuous agitation (200 rpm) followed by an adsorption process with Amberlite® XAD-16. The esterification of some hydroxycinnamic acids can occur at different positions of quinic acid moiety, resulting in four positional isomers. The fragment at m/z 191 found in compounds from this class corresponds to the loss of quinic acid.

Peaks with the $[M-H]^-$ at m/z 337 were identified as coumaroylquinic acids (CoQAs) [48]. In particular, 3-CoAs and 4-CoAs were found in Saco cherry antioxidant polyphenol-rich extracts and showed the typical fragments of the *p*-coumaric acid m/z 163 and m/z 119 [49] and the loss of quinic acid. Moreover, other classes of CGAs were found: Feruloylquinic acids (FQAs) with a negative molecular at $[M-H]^-$ m/z 367, and diCQAs with a negative molecular at $[M-H]^-$ m/z 515. A total of 23 hydroxycinnamic acids were identified in Saco extracts, showing that this class predominates in this fruit, being that these data are supported by other studies performed in the cherries from this variety [4,8,41,47]. Since it has been recognized that CGAs can have health benefits, these findings in the Saco cherry extracts provide insight into the knowledge of the profile of these phenolic compounds group in sweet cherry [50].

Regarding hydroxybenzoic acids, SC cherry extracts present 5 compounds. The protocatechuic acid and one derivative were found with a m/z 315 were observed, and in the MS^2 experiments of the derivative of protocatechuic acid gave a base peak at m/z 153, corresponding to protocatechuic acid aglycone that corresponds to a loss of 162 Da [5]. One signal at m/z 299 gave a base peak in the fragmentation spectra at m/z 137, which is indicative of the presence of a hydroxybenzoic acid residue. The peak 32 showed a molecular negative ion at m/z 329, which fragmented in the MS^2 experiments giving a base peak at m/z 167, suggesting the presence of a vanillic acid residue. The absence of sugar fragmentation evidence prompts us to tentatively identify this compound as vanillic acid, but regarding the m/z, these compounds could be a vanillic acid-glycoside. These 6 hydroxybenzoic acids were already documented in sweet cherries from exotic varieties, such as Della Marca, Celeste, Bigarreau, Durone Nero, Lapins, and Moretta by Martini, Conte, and Tagliazucchi [5], and one hydroxybenzoic acid derivative was identified in Saco cherries [47]. However, compounds of this class were not reported in the Saco cherry (poly)phenol-rich extracts produced according to Matias, Rosado-Ramos, Nunes, Figueira, Serra, Bronze, Santos, and Duarte [8].

Table 3. LC-ESI-UHR-QqTOF-MS data of phenolic compounds in different extracts from non-compliant Saco cherry.

Proposed Compound	Molecular Formula	RT (min)	m/z calcd ([M-H]-)	Error (mDa)	Major Fragments Negative MS/MS Ions (m/z)	Saco Cherry Extracts			
						CE	CET	MAE	UAE
Hydroxycinnamic acids									
3-Caffeoylquinic acid *cis*	$C_{16}H_{18}O_9$	7.7	353.1	1.0	191(100), 179(80), 135(16)	D.	D.	D.	D.
3-Caffeoylquinic acid *trans*	$C_{16}H_{18}O_9$	7.8	353.1	1.3	191(100), 179(54), 135(28)	D.	D.	D.	D.
5-Caffeoylquinic acid *trans*	$C_{16}H_{18}O_9$	8.8	353.1	1.1	191.05(100)	D.	D.	D.	D.
4-Caffeoylquinic acid *trans*	$C_{16}H_{18}O_9$	9	353.1	1.2	173(100), 179(70), 191(40), 135(20)	D.	D.	D.	D.
3-Coumaroylquinic acid	$C_{16}H_{18}O_8$	8.6	337.1	1.3	163.(100), 119(18), 191(10)	D.	D.	D.	D.
4-Coumaroylquinic acid *cis*	$C_{16}H_{18}O_8$	9.8	337.1	0.6	173(100), 163(16), 191(15)	D.	D.	D.	D.
4-Coumaroylquinic acid *trans*	$C_{16}H_{18}O_8$	10.2	337.1	1.1	173(86), 163.03(24)	D.	D.	D.	D.
Feruloylquinic acid isomer	$C_{16}H_{16}O_{10}$	6,9	367.1	1.1	163(18), 205(16)	D.	D.	D.	D.
3-Feruloylquinic acid *cis*	$C_{17}H_{20}O_9$	8.4	367.1	0.9	193(100), 134(10), 149(3)	D.	D.	D.	D.
5-Feruloylquinic acid *cis*	$C_{17}H_{20}O_9$	10.1	367.1	0.8	173(100)	D.	D.	N.D.	D.
Caffeoylquinic acid-glycoside	$C_{22}H_{27}O_{14}$	6.4	515.1	1.6	341(35), 179(27), 335(11), 191(8), 353(6), 323(3)	D.	D.	D.	D.
4,5-diCaffeoylquinic acid	$C_{25}H_{24}O_{12}$	12.9	515.1	0.7	335(91), 191(32), 179(27),	D.	D.	N.D.	D.
4-Caffeoylquinic acid lactone	$C_{16}H_{16}O_8$	10.5	335.1	1.0	161(77), 191(15), 135(15)	D.	D.	D.	D.
4-Coumaroylquinic acid lactone	$C_{16}H_{16}O_7$	12.2	319.0	1.1	145(100), 119(14), 163(9), 173(4)	D.	D.	D.	D.
p-Coumaric acid derivative	$C_9H_8O_3$	6.9	163.0	0.8	119(100)	D.	D.	D.	D.
Coumaroyl hexose	$C_{15}H_{18}O_8$	8.2	325.1	0.8	145(100), 163(18), 265(4.5), 187(3), 205(3)	D.	D.	D.	D.
Caffeic acid-glycoside	$C_{15}H_{17}O_9$	8.7	341.1	0.9	179(100), 135(13)	D.	D.	D.	D.
Caffeoyl alcohol 3/4-o-hexoside	$C_{15}H_{19}O_8$	8.5	327.1	0.7	165(100), 121(2)	D.	D.	D.	D.
Feruloyl hexose	$C_{16}H_{19}O_9$	9.6	355.1	1.5	175(100), 193(46), 295(8)	D.	D.	D.	D.
Sinapoyl hexose	$C_{17}H_{21}O10$	8.1	385.1	1.1	223(100), 208(12)	D.	D.	D.	D.
Flavanols									
Epicatechin-3-gallate	$C_{22}H_{18}O_{10}$	12.8	441.1		395(100), 263(81), 441(6)	D.	D.	D.	D.
Flavonols									
Quercetin-7-O-glucoside-3-O-rutinoside	$C_{33}H_{40}O_{21}$	10.5	771.2	1.4	609(100)	D.	D.	D.	D.
Kaempferol-3-glucoside	$C_{21}H_{20}O_{11}$	17.6	447.1	1.2	285(92)	D.	N.D.	D.	N.D.

Table 3. *Cont.*

Proposed Compound	Molecular Formula	RT (min)	m/z calcd ([M-H]-)	Error (mDa)	Major Fragments Negative MS/MS Ions (m/z)	Saco Cherry Extracts			
						CE	CET	MAE	UAE
Kaempferol-3-rutinoside	$C_{27}H_{30}O_{16}$	12.5	593.1	1.7	285(29)	D.	D.	D.	D.
Other flavonoids									
Taxifolyn-rutinoside	$C_{27}H_{31}O_{16}$	9	611.1	2.2	285(93), 475(76), 501(17), 241(13), 485(10), 303(4)	D.	D.	D.	D.
Hydroxybenzoic acids									
Protocatechuic acid-glycoside	$C_{13}H_{15}O_9$	6.3	315.1	0.6	109(5), 153(100)	D.	D.	D.	D.
Protocatechuic acid	$C_7H_6O_4$	7,2	153.0	0.1	109(100)	D.	D.	D.	D.
Hydroxybenzoic acid-glycoside	$C_{13}H_{16}O_8$	6,3	299.1	0.9	137(100)	D.	D.	D.	D.
Hydroxybenzoyl hexose	$C_{13}H_{16}O_8$	7.4	299.1	1.0	137(29), 179(20), 239(14)	D.	D.	D.	D.
Vanillic acid-glycoside	$C_{14}H_{17}O_9$	7.2	329.1	0.6	167(100)	D.	D.	D.	D.
Anthocyanins									
Cyanidin-3-glucoside	$C_{21}H_{21}O_{11}$	12.3	449.2	0.1	287(100)	D.	D.	D.	D.
Cyanidin-3-rutinoside	$C_{27}H_{31}O_{15}$		595.1	0.6	287(100), 449(14)	D.	D.	D.	D.
Peonidin-3-glucoside	$C_{22}H_{23}O_{11}$	12.4	463.1	0.6	301(100)	D.	D.	D.	D.
Peonidin-3-rutinoside	$C_{28}H_{33}O_{15}$	11.4	609.1	1.0	301(100), 463 (15)	D.	D.	D.	D.
Pelargonidin-3-rutinoside	$C_{27}H_{31}O_{14}$	14	579.1	1.3	271(100), 433 (20)	D.	N.D.	D.	D.

Note: N.D, non-detectable; D., detectable; RT, retention time; m/z calcd ($[M-H]^-$): Experimental values.

Epicatechin-3-gallate (m/z 441.1), previously described in cherries, was the only compound identified within the flavanols class. This fact was not surprising, since Gonçalves, et al. [51] observed that the compounds from the same class decreased in Saco variety during storage time. Additionally, Acero, Gradillas, Beltran, García, and Mingarro [1] did not find any compound from flavanols in sweet cherries cultivated in Spain. On the other hand, Martini, Conte, and Tagliazucchi [5] identified 12 more flavanols in cherry exotic varieties, including procyanidins, and Serra, Duarte, Bronze, and Duarte [4] identified two flavanols in Saco cherries ((+)-catechin and (−)-epicatechin). Furthermore, Matias, Rosado-Ramos, Nunes, Figueira, Serra, Bronze, Santos, and Duarte [8] reported the presence of (+)-catechin and procyanidin B2 in antioxidants extracts from Saco cherry produced by recovery (poly)phenols from extracts by adsorption process, although, Gonçalves, Rodrigues, Santos, Alves, and Silva [17] did not identify any flavanols in Saco cherry hydro-alcoholic extracts.

Among flavonols, only 3 derivatives were identified: Kaempferol-3-rutinoside, kaempferol-3-glucoside, and quercetin derivative, which were reported previously by Martini, Conte, and Tagliazucchi [5] in exotic varieties and Portuguese varieties, for instance, Saco sweet cherry for Gonçalves, Bento, Silva, and Silva [47], and in Saco cherry polyphenol-rich extracts [8,17]. Regarding anthocyanins, a total of 5 were identified in antioxidant polyphenol-rich extracts: Cyanidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-rutinoside, peonidin-3-glucoside, and pelargonidin-3-glucoside. These anthocyanins had been already identified in the Saco variety [47] and in polyphenol-rich extracts produced from Saco cherry [17], being that these anthocyanins are the most reported in cherries, in spite of the variety. All the extraction methodologies showed to be able to recover all phenolic compounds except on MAE extracts, where three of the hydroxycinnamic acids were not detected. Furthermore, only with CET was it not possible to recover all the mainly identified anthocyanins in sweet cherry, because pelargonidin-3-rutinoside was not present in this extract. Elevated temperatures have been reported to improve the efficiency of extraction due to enhanced diffusion rate and solubility of analytes in solvents. However, elevated temperature for long periods may increase the anthocyanins degradation rate because the degradation rate of anthocyanins is time and temperature-dependent. Therefore, high-temperature in short-time extraction conditions and processing treatments have been used successful in retarding anthocyanin degradation in fruits. This could explain the recovery of all anthocyanins in MAE, but not in CET. For instance, research carried out by Ju and Howard [51] reports that optimal MAE conditions differed for anthocyanins and phenolic acids, especially in terms of temperature and irradiation time, so lower temperature (<60 °C) and shorter time were more convenient for anthocyanins extraction. On the other hand, for phenolic acids, higher extraction yield at higher temperatures (70 °C) and longer irradiation time (10 min) was observed.

3.3.2. Quantification of Phenolic Compounds by HPLC-DAD

The individual phenolic compounds of Saco cherry extracts quantified using HPLC-DAD are summarized in Table 4. The extracts, despite the differences observed in the amounts of each phenolic compound, exhibited similar chromatographic profile, as expected, due to the previous identification reported (Section 3.3.1). The anthocyanins were the most representative class with the highest amount present in cherry extracts being cyanidin-3-rutinoside, the major compound identified in all extracts represented, approximately 70% of the total content of anthocyanins and 40% of total phenolic compounds quantified. The CET and MAE extracts showed the highest amount of this anthocyanin ($p > 0.05$), 2.54 ± 0.16 and 2.46 ± 0.03 mg/DE, respectively). However, MAE allowed for the recovery of higher amounts of cyanidin-3-glucoside (1.14 ± 0.01 mg/g DE), peonidin-3-rutinoside (0.16 ± 0.03 mg/g DE) ($p > 0.05$), and pelargonidin-3-rutinoside (0.06 ± 0.05 mg/g DE) than the other extraction methodologies. Similar results were reported by other authors where the main anthocyanins in sweet cherry were cyanidins, peonidin-3-rutinoside, and pelargonidin-3-rutinoside [3]. Therefore, our data is in accordance with previous works on Saco cherries, where Gonçalves, Bento, Silva, and Silva [47], and Serra, Duarte, Bronze, and Duarte [4] reported cyanidin-3-O-rutinoside and

cyanidin-3-O-glucoside as the main anthocyanins present in sweet cherries from Saco variety and also quantified the two anthocyanins reported in Saco cherry. On the other hand, a study performed by Gonçalves, Rodrigues, Santos, Alves, and Silva [17] reported that a Saco cherry extract produced with conventional extraction followed by a C18 solid-phase extraction (SPE) is rich in the same anthocyanins, however, the amount of anthocyanins is higher because SPE allows purification and consequently increased extract concentration. Additionally, a (poly)phenol-rich extract was produced by Matias, Rosado-Ramos, Nunes, Figueira, Serra, Bronze, Santos, and Duarte [8] with a two-step separation process: First encompasses a solid-liquid CE followed by an adsorption process, showed higher amount of cyanidin-3-rutinoside, cyanidin-3-glucoside, and peonidin-3-glucoside, however, pelargonidin-3-rutinoside was not present. The great diversity of phenolic compounds found in Saco cherry highlights the importance to valorise non-compliant fruit because although it does not have acceptable parameters for direct sale in fresh food chains, it is still rich in BCs such as phenolic compounds. Furthermore, the valorisation of the regional fruit variety supports the preventing of habitat loss due to urbanization and provides a boost to the local bioeconomy by promoting the commercialization of new products deriving from regional varieties, and thereby reduces the C footprint in the environment due to the valorisation of food losses [2].

Table 4. Quantification of main phenolic compounds (mg/g DE (Dry extract)) in different extracts from Saco cherry by HPL-DAD. Values are represented by the average ± standard deviation. Different letters mean significant differences between extraction techniques, determined by Tukey's test ($p < 0.05$).

Phenolic Compounds	Saco Cherry Extracts			
	CE	CET	MAE	UAE
Hydroxycinnamic acids				
3-Caffeoylquinic acid	0.54 ± 0.05 ^{c}	0.87 ± 0.02 ^{b}	0.99 ± 0.07 ^{a}	0.54 ± 0.00 ^{c}
5-Caffeoylquinic acid	0.40 ± 0.08 ^{ab}	0.26 ± 0.05 ^{c}	0.38 ± 0.01 ^{b}	0.46 ± 0.01 ^{a}
4-Caffeoylquinic acid	B.Q.L	0.06 ± 0.03 ^{b}	0.15 ± 0.04 ^{a}	0.09 ± 0.02 ^{a}
4,5-dicaffeoylquinic acid	0.08 ± 0.02 ^{a}	0.08 ± 0.00 ^{a}	N.D	0.02 ± 0.00 ^{b}
Caffeic acid	B.D.L	0.09 ± 0.03 ^{a}	0.08 ± 0.02 ^{a}	B.Q.L
p-coumaroylquinic acid	0.47 ± 0.20 ^{c}	1.03 ± 0.05 ^{a}	0.63 ± 0.00 ^{b}	0.39 ± 0.00 ^{d}
p-coumaric acid	0.20 ± 0.03 ^{c}	B.Q.L	0.62 ± 0.05 ^{a}	0.31 ± 0.00 ^{b}
Flavan-3-ols				
(−)-Epicatechin-3-gallate	B.Q.L	B.Q.L	0.03 ± 0.08 ^{a}	B.Q.L
Flavonols				
Quercetin-3-rutinoside	0.42 ± 0.63 ^{a}	0.24 ± 0.02 ^{b}	0.14 ± 0.00 ^{c}	0.05 ± 0.01 ^{d}
Kaempferol-3-glucoside	0.07 ± 0.00 ^{a}	N.D.	0.06 ± 0.02 ^{b}	N.D.
Quercetin	B.D.L.	B.D.L.	0.12 ± 0.00 ^{a}	B.D.L.
Anthocyanins				
Cyanidin-3-rutinoside	2.13 ± 0.25 ^{b}	2.54 ± 0.16 ^{a}	2.46 ± 0.13 ^{a}	2.21 ± 0.19 ^{b}
Cyanidin-3-glucoside	0.58 ± 0.04 ^{c}	0.87 ± 0.11 ^{b}	1.14 ± 0.01 ^{a}	0.54 ± 0.06 ^{c}
Peonidin-3-rutinoside	0.08 ± 0.00 ^{b}	0.07 ± 0.05 ^{b}	0.16 ± 0.03 ^{a}	0.06 ± 0.01 ^{b}
Pelargonidin-3-rutinoside	0.06 ± 0.01 ^{a}	N.D.	0.06 ± 0.05 ^{a}	0.04 ± 0.00 ^{a}

Abb: B.D.L., bellow detection limit; B.Q.L., bellow quantification limit; N.D., non-detectable.

Regarding the hydroxycinnamic acids in SC cherry extracts, 3-caffeoylquinic acid, 5-caffeoylquinic acid, *p*-coumaroylquinic acid, and *p*-coumaric acid were the major compounds in extracts. These compounds were found in greater quantities in extracts obtained by MAE (0.99 ± 0.07, 0.38 ± 0.01, 0.63 ± 0.00, and 0.62 ± 0.05 mg/g DE, respectively) and CET (0.87 ± 0.02, 0.26 ± 0.05, and 1.03 ± 0.05, respectively), with the exception of 5-caffeoylquinic acid (UAE recovery the higher amount (0.46 ± 0.01 mg/g DE)) and *p*-coumaric acid (the concentration in CET was below the quantification limit). Nevertheless, extracts produced by Matias, Rosado-Ramos, Nunes, Figueira, Serra, Bronze, Santos, and Duarte [8], and Gonçalves, Rodrigues, Santos, Alves, and Silva [17] with two-step separation process also had more quantity of hydroxycinnamic acids (likewise anthocyanins), but they present less diversity of compounds.

The epicatechin-3-gallate was only detected in MAE extract (0.02 ± 0.08 mg/g DE), and among the flavonols, the higher amount was found in CE extract with 0.42 ± 0.63 mg/g DE of quercetin-3-rutinoside

and 0.05 ± 0.00 mg/g DE, however, quercetin-3-glucoside was only quantified on MAE extract (0.12 ± 0.00 mg/g DE). The MAE, likewise, observed in TPC showed a higher amount of phenolic compounds.

Although HPLC-DAD analysis is a well-documented technique for polyphenols quantification, the sum of all phenolic compounds quantified was lower than those quantified by Folin–Ciocalteu assay (Table 2) for all the extracts. These differences, despite some of the minor peaks, could not be quantified, because they were under detection range given the lowest values of quantification, the use of Folin–Ciocalteu's reagent can also react with polysaccharides and proteins given an overestimation of total phenolic content [46]. Furthermore, the TPC results are expressed based in gallic acid equivalents and on HPLC results, and it is quantified with the exact amount of phenolic compound itself.

3.4. Antioxidant Activity

Natural antioxidants present in fruits have gained interest among the scientific community, consumers, and food industries since epidemiological studies have indicated that regular consumption of natural antioxidants is associated with a lower risk of chronic diseases and could replace synthetic antioxidants in foodstuffs [52]. To evaluate the antioxidant activity of functional extracts, different methods with different action mechanisms need to be used. Since the antioxidant capacity of food is determined by a mixture of different phenolic compounds with different action mechanisms, among which synergistic interactions are also occurring, it is necessary to combine more than one method in order to determine in vitro antioxidant capacity [53]. Stable radicals such as ABTS or DPPH are among the most popular colorimetric methods to evaluate antioxidant activity in foods. Nevertheless, ABTS applies to both hydrophilic and lipophilic antioxidant systems, whereas DPPH applies to hydrophobic systems [54]. ORAC method has been reported by different authors as a more relevant antioxidant procedure because it engages a biological radical (peroxyl) source and is a very high sensitivity method, which is mainly used to measure the activity of hydrophilic antioxidants compounds [23,55].

Data of in vitro antioxidant activity measured by ORAC, ABTS, and DPPH methods are shown in Figure 2. In Saco cherry extracts, antioxidant activity measured by ORAC, ABTS°, and DPPH° methods ranged from 35.62 ± 4.80 to 50.46 ± 4.80 TE/g DE, from 6.73 ± 0.38 to 10.88 ± 0.38 AAE/g DE, and from 6.66 ± 0.32 to 9.58 ± 0.42 mg TE/g DE, respectively. The SC cherry extracts had the highest antioxidant capacity and exhibited good scavenging effects on ORAC assay using MAE, presenting values of 50.46 ± 1.58 mg TE/g DE, followed by CE (38.34 ± 4.09 mg TE/g DE), UAE (35.62 ± 4.80 mg TE/g DM), and CET (35.80 ± 2.18 mg TE/g DE), without significant differences ($p > 0.05$) between the last three extraction methods. The ORAC values of Saco cherry showed a high positive correlation ($R^2 = 0.9853$) with the previous values of TPC. The values obtained by the ORAC method were much higher than the values by DPPH° and ABTS°. Similar differences were reported in antioxidant assays of several fruit, plants, and by-products [56]. These differences probably arise from the different mechanisms involved during each methodology: Single electron transfer (SET) in the case of DPPH/ABTS, and hydrogen atom transfer (HAT) in the case of the ORAC assay [56].

Regarding ABTS° assay results, the Saco cherry extracts obtained by MAE also presented higher antioxidant activity (10.88 ± 0.38 mg AAE/g DE), followed by CE (7.80 ± 0.56 mg AAE/g DE) and CET (7.65 ± 0.28 mg AAE/g DE), and with the lowest values attributed by UAE (6.73 ± 0.38 mg AAE/g DE).

The inhibition of DPPH° free radicals was similar for Saco cherry extracts obtained by MAE (9.58 ± 0.42 mg TE/g DE) and UAE (9.44 ± 0.38 mg TE/g DE) ($p > 0.05$), however, they had more scavenging activity than extracts obtained by the conventional methods with low and high temperature, 6.98 ± 0.55 and 6.66 ± 0.32 mg TE/g DE, respectively. In Saco cherry extracts, the DPPH° method did not correlate with the TPC ($R^2 = 0.4099$). This could be due to the fact that the DPPH° method used a free radical that can only be dissolved in an organic solvent and therefore evaluates hydrophobic systems [54]. The higher antioxidant activity obtained on SC cherry extracts (observed in all different antioxidant assays) through the application of MAE, could be the result of a better plant cells disruption, as reported for this methodology. As already stated, these extracts have more phenolic compounds,

and this extraction technique makes compounds more bio-accessible, mainly anthocyanidins and flavonols, which have been reported to have more scavenging activity than most common phenolic compounds, such as phenolic acids, due to the great number of functional groups (OH^-) present in their structure [41,57].

There are no specific studies about the use of non-compliant cherries to produce functional extracts with an antioxidant activity using green methodologies such as MAE and UAE. Notwithstanding, there are some studies about the antioxidant activity of Saco cherry fruit, namely Gonçalves, Bento, Silva, and Silva [47], reporting that this variety exhibited a dose-dependent effect against DPPH° radical, and Serra, Duarte, Bronze, and Duarte [4] reported that Saco cherry showed higher values of ORAC than other Portuguese cherry varieties. There are also three important studies about the Saco cherry extract production reporting (1) that a polyphenol extract produced by conventional hydro-alcoholic extraction with low temperature was more active than the extract fractions obtained that followed concentration and purification with SPE column [17]; (2) the (poly)phenol-rich extract obtained by a CE with EtOH:H2O (1:1 *v*/*v*) for 2 h followed an adsorption process with Amberlite® XAD-16 showed a higher antioxidant activity by ORAC method, being about two-fold higher than the control (Vitamin C) [8], and (3) Serra et al. [58] reported the use of supercritical CO_2 extraction followed by solvent extraction with CO_2:EtOH (90:10, *v*/*v*) and extracts revealed a great antioxidant activity. However, the differences between previous works and our results could be due to the differences in the extraction methodologies

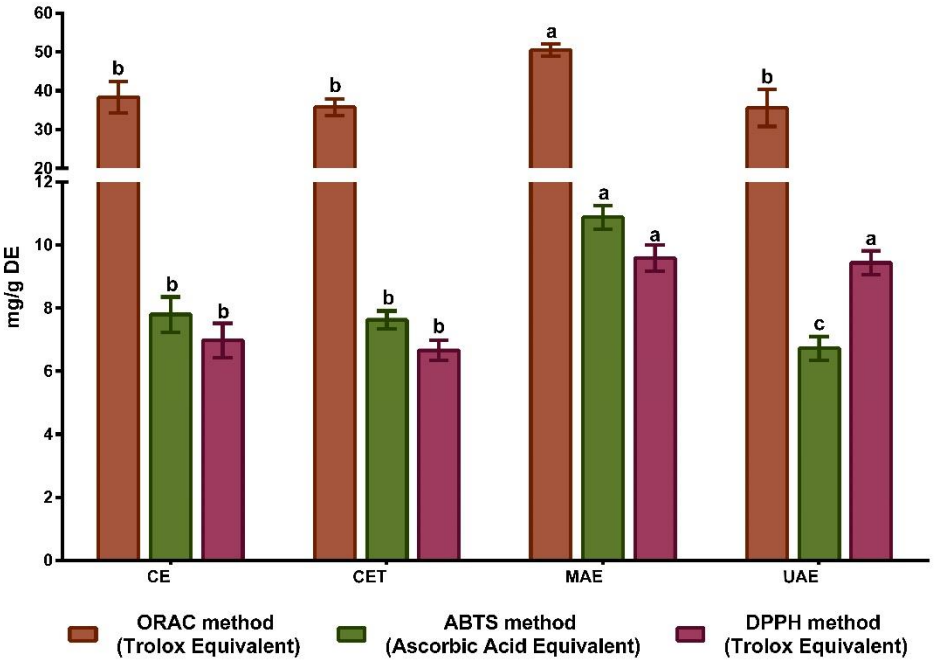

Figure 2. Antioxidant activity of different Saco cherry extracts by oxygen radical absorbance capacity (ORAC) method (expressed as mg TE (Trolox equivalent)/g DE), ABTS° (2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) method (expressed as mg AAE/g DE) and DPPH° (2,2-diphenyl-1-picrylhydrazyl) method (expressed as mg TE/g DE). Different letters mean significant differences between extraction techniques for each antioxidant activity assay (same colour in the graph), determined by Tukey's test ($p < 0.05$).

Overall, some of the phenolic compounds identified in Saco cherry extracts are well-documented as efficient reducing compounds and scavenging free radicals, and furthermore has been demonstrated as being more effective than other BCs in fruit. Regarding anthocyanins, several studies showed that these compounds have potential to inhibit tumour growth, slow cardiovascular diseases, and retard the ageing process due to their higher antioxidant activity. Additionally, other flavonoids

identified in cherry extracts, such as quercetin, catechin and epicatechin, neochlorogenic acid, and chlorogenic acid are widely reported to act as powerful antioxidants and anticancer agents. Moreover, it is important to reinforce the possible presence of other non-determined reducing compounds, such as carotenoids, organic acids, and volatile compounds, which may also contribute to increasing the total antioxidant activity values obtained by chemical in vitro methods [17], and that were not determined and quantified in the antioxidant extracts produced.

Nevertheless, this relevant evidence on these fruit extracts shows the importance of valorising non-compliant fruit as natural sources of BCs, mainly antioxidant compounds, being a low-cost resource that could be used to develop natural antioxidant extracts to apply in the food industry. On the other hand, the production of natural antioxidant extracts from non-compliant fruit represents a solution with low environmental impact since it uses food that had not added-value.

3.5. Principal Component Analysis

In order to achieve a better overview, due to the large amount of data obtained, to distinguish the different antioxidant extracts according to their phenolic compounds content and antioxidant activity. and to identify a potential relationship between the extraction techniques applied, a principal component analysis (PCA) was performed for the Saco cherry extracts. The 20 variables considered in PCA are described in Table 5.

Table 5. Description of the 20 variables considered in principal component analysis (PCA) for Saco cherry extracts.

Saco Cherry Extracts			
Variable Number	**Variable Designation**	**Variable Number**	**Variable Designation**
1	Total Phenolic Content	11	5-Caffeoylquinic acid
2	Total Anthocyanins Content	12	4-Caffeoylquinic acid
3	ABTS assay	13	4,5-Di-O-Caffeoylquinic acid
4	DPPH assay	14	Caffeic acid
5	ORAC assay	15	*p*-coumaroylquinic acid
6	Cyanidin-3-rutinoside	16	*p*-coumaric acid
7	Cyanidin-3-glucoside	17	Epicatechin-3-gallate
8	Peonidin-3-rutinoside	18	Quercetin-3-rutinoside
9	Pelargonidin-3-rutinoside	19	Kaempferol
10	3-Caffeoylquinic acid	20	Quercetin

The cumulative percentage of the total variance explained by the first two components was 85.2% (Figure 3), where the PCA bidimensional plot shows the information about samples (dots) and variables (vectors) as a data matrix. Component 1, which described 52.44% of parameters variability, was positively influenced by almost ten variables, such as TPC, antioxidant activity (ABTS° and ORAC method), 3-caffeoylquinic acid, peonidin-3-rutinoside, and pelargonidin-3-rutinoside. Component 2, which accounted for 32.78% of the variability, received the main positive contribution from 5-caffeoylquinic acid, antioxidant activity (DPPH° method), pelargonidin-3-rutinoside, and *p*-coumaric acid. By the analysis of the variables, it was possible to verify that the TPC is more correlated with ABTS° and ORAC than DPPH°, and the flavonols and anthocyanins are more correlated with antioxidant activity due to their structure since they have higher antioxidant potential than other phenolics groups. MAE is represented in the positive values of the components due to a higher number and levels of anthocyanins and phenolics as well as great antioxidant activity; CET is represented in the negative values of component 2 due to a lower number of anthocyanins as well as antioxidant activity. On the positive side of component 2, it can be seen that CE and UAE, which are related with a lower amount of anthocyanins, are the main compounds in sweet cherry responsible for the high values of antioxidant activity.

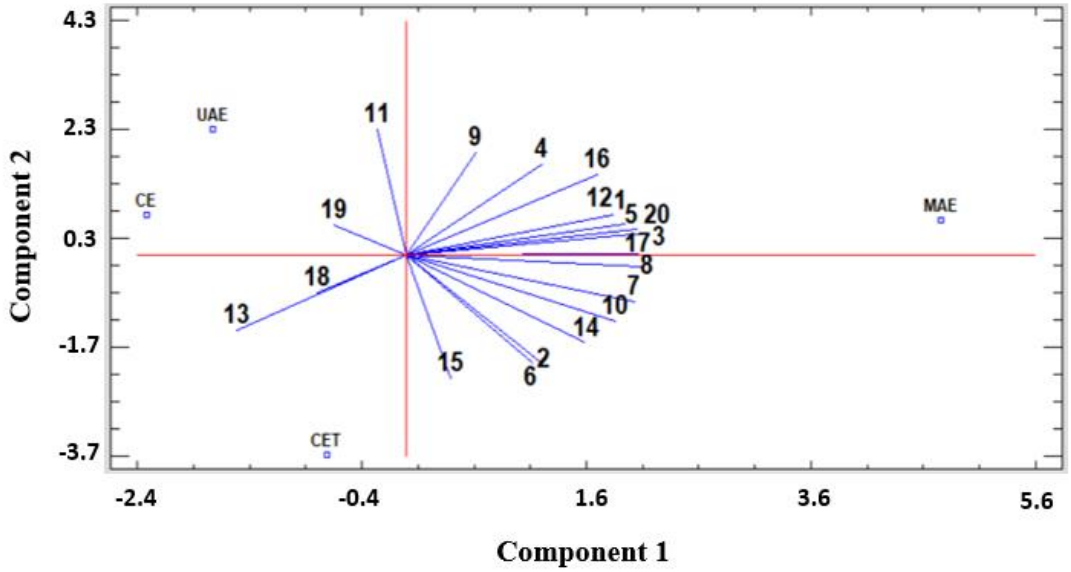

Figure 3. Principal Component Analysis (PCA) biplot for the 20 parameters analysed in Saco cherry extracts. The first component explained 52.44% of the variance and the second component explained 32.78%.

The PCA confirms all the results discussed previously, concluding that the use of MAE was the most efficient and the use of CET using 65 °C presented the lowest values of phenolic compounds as well as antioxidant activity. These results are in line with expectations since the exposure of anthocyanins to high temperatures over long periods causes their degradation. Therefore, application of MAE for 15 min allowed us to obtain polyphenol-rich extracts with better antioxidant capacity than UAE applied for 30 min, or CE and CET for 30 min. The advantages of MAE are that high-efficiency extraction can be done using only the water content present in cherry, while simultaneously reducing extraction time. Besides, easy maintenance of extraction vessels, enhancement of recovery and repeatability, and the possibility of simultaneous extraction of multiple samples are also better with MAE than with CE techniques and UAE [40,59].

4. Conclusions

In the present work, different extraction methodologies (CE, CET, MAE, and UAE) were applied to develop polyphenolic-rich extracts aiming at developing new functional ingredients with antioxidant activity using non-compliant sweet cherry. The results showed that MAE is better than conventional extraction methods (CE and CET) and the other green methodology applied (UAE) to obtain functional extracts from non-compliant Saco cherry. With this green solid-liquid extraction technique, it was possible to obtain a polyphenolic-rich extract with the highest ABTS, DPPH, and ORAC values. The main phenolic compounds identified included neochlorogenic acid, *p*-coumaric, quercetin-3-rutinoside, and anthocyanins, namely cyanidin-3-rutinoside. The current results proved that the non-compliant Saco cherry are indeed of high value to be used as sources of functional ingredients extraction, which might be useful for human consumption. The application of new alternative solutions for the valorisation of non-compliant cherries brings value to the fruit supply chain, using all fruit produced, since the obtained extracts had high percentages of functional ingredients. Furthermore, on a longer-term perspective, these results sensitize to the preservation of the traditional and local sweet cherry variety. Therefore, it is possible, based on the richness of BCs present in low calibre and non-compliant cherries, to obtain a diversity of new ingredients for the food industry, minimizing environmental impact, promoting the bioeconomic value of the fruit, and promoting sustainability.

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