Comparison of Extraction Techniques for the Recovery of Sugars, Antioxidant and Antimicrobial Compounds from Agro-Industrial Wastes

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Abstract: Agro-industrial wastes can be used to obtain high-value compounds rich in antioxidant and antimicrobial activity. This study aimed to compare different extraction techniques for the recovery of sugars, antioxidants, and antimicrobial compounds from brewer’s spent grain (BSG), blue agave bagasse (BAB), spoiled blackberries (BB), and raspberries (RB). Aqueous (AQ), enzymatic (E), chemical-enzymatic (CE), and hydroalcoholic (EOH) extractions were assessed, and sugars, phenolics, flavonoids, and anthocyanin contents were quantified. Antioxidant activity of the extracts was evaluated using the ABTS and DPPH assays, and antimicrobial activity was tested against three yeasts and six bacteria. The CE process gave the highest total and reducing sugars content for the four residues tested, and the highest antioxidant activity, phenolics, flavonoids and anthocyanin content for BAB and BSG. Regarding BB and RB, the best treatment to obtain total and reducing sugars and antioxidant activity with ABTS was CE; the highest content of anthocyanins, phenolic, flavonoids and antioxidant activity with DPPH was obtained with EOH treatment. CE extracts of BSG and RB showed the highest inhibition against the strains studied. Results show that BSG, BB, and RB can be a source of antioxidants and antimicrobial compounds for the food and pharmaceutical industries. Depending on the desired application and component of interest, one of the extraction techniques evaluated here could be used.

Keywords: brewer’s spent grain; blue agave bagasse; berries; extracts; hydrolysates

1. Introduction

Agriculture was responsible for the global production of 9.2 billion tons of primary crops in 2018, being the main source of food worldwide [1]. However, food production generates large amounts of waste during post-harvest processing and consumption, as well as degraded and contaminated products that are not suitable for human consumption [2,3]. Agro-industrial wastes can be revalorized using different strategies. For example, such wastes might be can be used as a low-cost feedstock for biorefineries, as a source of high-value compounds like antioxidants and antimicrobial agents, or, since they contain compounds, such as carbohydrates, lipids, and lignin, they can be converted into various products, such as biochemicals, biomass, biofuels, and enzymes [2,4].

In Mexico, agricultural production reached 264.6 million tons in 2020 [5], with nearly 20 million tons of food wasted per year [6]. Specifically, Jalisco is the largest agricultural producer in Mexico, with a production over 40 million tons per year [7]. It is currently the largest producer of Agave tequilana, blueberry, raspberry, corn, and milk, and the second-largest producer of avocado, sugar cane, safflower, coconut, watermelon, and blackberry [8].
In addition, Jalisco has several processing industries, including tequila, craft breweries, dairy, and the production and marketing of berries, where raspberries and blackberries stand out. All these industries generate different types of waste that can be revalorized to recover carbohydrates and bioactive compounds with antioxidant and antimicrobial activities, including phenolic compounds, flavonoids, and anthocyanins.

Hydrolysates of BAB and BSG have been used as low-cost substrates to produce compounds, such as yeast biomass, enzymes, single-cell proteins, single-cell lipids, carotenoids, organic acids, and ethanol [4,9–14]. As for antioxidants, they are of interest since they can prevent damage in the organisms provoked by oxidant species. Polyphenols are natural antioxidants that have beneficial effects on human health. For instance, their consumption decreases the incidence and progression of chronic, neurodegenerative, and cardiovascular diseases, as well as preventing tumor growth and cancer [15]. Furthermore, phenolic compounds also present antimicrobial activity [16], so they can be employed as additives to prevent oxidation and food spoilage [17].

High value-added compounds cannot be directly obtained. Therefore, agro-industrial wastes must be subjected to different treatments to recover target molecules from the different matrices. Technologies can be chemical, physical, or biological, and must be adapted to the residue used.

For example, lignocellulosic materials need acid or alkali pretreatments in order to decrease cellulose crystallinity, remove lignin and improve the residue porosity [18]. This helps to enhance the enzymatic hydrolysis and recovery of the desired components. Residues, such as agave bagasse and brewer’s spent grain have been treated through chemical and enzymatic hydrolysis to recover carbohydrates and antioxidants [13,14,19,20]. In fruits, several methods have been tested to reduce the degradation of phenolic compounds during extraction [21–23]. Therefore, it is necessary to evaluate the impact of the extraction methods on the compounds of interest, so that the different types of residues can be used as sources of natural antioxidant and antimicrobial agents.

Due to the high availability of agro-industrial waste in the region, and the increasing interest in their revalorization, blue agave bagasse (BAB) from the tequila industry, brewer’s spent grain (BSG) from craft beer production, raspberries (RB), and blackberries (BB) were selected for the recovery of sugars and compounds with antioxidant and antimicrobial activities, using four different extraction treatments.

2. Materials and Methods
2.1. Materials
2.1.1. Enzyme and Chemicals

Cellic® CTec2 was acquired from Novozyme (Bagsvaerd, Denmark), while 3,5-dinitrosalicylic acid (DNS), glycine, glucose, xylose, fructose, phenol, sulfuric acid, ninhydrin, and Folin-Ciocalteu reagent were bought from Sigma-Aldrich (Toluca, Mexico). Unless stated, other chemicals and solvents were of the highest purity available, and these were also purchased from Sigma-Aldrich.

2.1.2. Agro-Industrial Residues

Blue agave bagasse (BAB) and brewe r’s spent grain (BSG) were donated by a local tequila factory and brewery, respectively. The selected fruits do not have the characteristics required for commercialization, so these blackberries (BB) and raspberries (RB) were obtained from fruit discarded in the local market. The BSG was dried in an oven at 70 °C for 72 h. The BAB and dry BSG were ground and sieved, and the 40-mesh particle size fractions were used for the extractions. The BB and RB were mashed without water and processed with the extraction techniques. A chemical analysis of the BAB, BSG, BB, and RB extracts was carried out.
2.2. Methods

2.2.1. Characterization of Residues, Hydrolysates, and Extracts

The humidity content of the residues was measured using a moisture analyzer (ML-50, A&D Company, Tokyo, Japan) at a constant temperature of 105 °C until constant weight was achieved. Lignin content of the residues was determined according to NREL methodology. Ash, protein, lipid, and total dietary fiber contents were determined using the AOAC methodology [24]. For the hydrolysates and extracts, total sugars (TS) and reducing sugars (RS) were quantified using the phenol sulfuric acid method and 3,5-dinitrosalicylic acid (DNS) methodology, respectively [4]. All measurements regarding residue characterization were carried out in duplicate.

2.2.2. Extraction Techniques

Residue samples at 10% (w/v) were treated with 4 extraction methods at 50 °C and 220 rpm for 20 h. The strategies evaluated were as follows: (I) aqueous extraction (AQ); (II) enzymatic hydrolysis (E) with Cellic® CTec2 at 2% (v/v) in acetate buffer 50mM and pH 5.5; (III) chemical-enzymatic extraction (CE) by pretreating at 121 °C for 15 min with a 1% (v/v) H₂SO₄ solution, before the mixture was allowed to reach room temperature and the pH was adjusted at 5.5 before enzyme addition; (IV) hydroalcoholic extraction (EOH) with 50% (v/v) water/ethanol.

The extraction technologies were selected since they can be considered as environmentally friendly. We want to propose methodologies that follow the green chemistry principles, using catalysts that cause minimal or no impact on the environment, and which provide mild operation conditions.

2.2.3. Phenolic Compounds Quantification

Total phenolic content was determined with the following methodology. First, 25 µL of the samples were mixed with 100 µL of distilled water, followed by 25 µL of Folin–Ciocalteu reagent (FCR). The samples were mixed and allowed to stand for 6 min before 250 µL of a 7% (p/v) Na₂CO₃ aqueous solution was added. Water was added to adjust the final volume to 600 µL and, after 90 min at room temperature in the dark, absorbance was measured at 760 nm. Phenolic content was determined using a gallic acid calibration curve (0–700 µg/mL), prepared under the same conditions and expressed as milligram of gallic acid equivalents (mg GAE) per gram of dry residue [25].

2.2.4. Flavonoids Quantification

Total flavonoid content was quantified as follows: 100 µL of appropriately diluted extracts were mixed with 400 µL of distilled water and 30 µL of 5% (p/v) NaNO₂. After 5 min, 30 µL of 10% (p/v) AlCl₃·6H₂O solution was added, mixed, and the samples were left to stand for 5 min. Then, 200 µL of NaOH 1 M were added. The reaction solution was mixed, kept for 15 min at room temperature in the dark, and then the absorbance was determined at 415 nm. Total flavonoid content was calculated using a rutin calibration curve (0–1 mg/L) and expressed as milligram of rutin equivalents (mg RE) per gram of dry residue [26].

2.2.5. Anthocyanin Quantification

Total anthocyanin content was determined using a pH differential method [24]. 350 µL of the samples were mixed with 350 µL of pH 1 potassium chloride buffer (25 mM), and 350 µL of the samples were mixed with 350 µL of pH 4.5 sodium acetate buffer (400 mM). The absorbances of both mixtures were measured at 520 nm and 700 nm, and the total anthocyanin content was calculated using the following equations:

\[
\text{Contents of anthocyanins (mg CGE/100 g residue DW)} = \frac{(A \times MW \times 1000 \times \text{dilution factor})}{(\varepsilon \times 0.56)}
\]

\[
A = [(\text{Abs}_{520} - \text{Abs}_{700})_{pH1.0}] - [(\text{Abs}_{520} - \text{Abs}_{700})_{pH4.5}]
\]
where ε is the molar extinction coefficient for cyanidin-3-glucoside (26,900 L/(mol cm)), MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), and 0.56 is the pathlength in cm. The results were expressed as milligrams of cyaniding-3-glucoside equivalents per 100 g of dry residue (mg CGE/100g residue DW).

2.2.6. Antioxidant Activity

Trolox equivalent antioxidant capacity (TEAC) was determined by ABTS radical scavenging assay as described by Moreira et al. [27] with some modifications. ABTS radical cation (ABTS⁺) was produced by reacting a 7.4 mM ABTS solution with a 2.45 mM potassium persulfate aqueous solution at a 1:1 (v/v) proportion and allowing the mixture to react under dark conditions at room temperature for 16 h. For the assessment of samples, the ABTS⁺ solution was diluted to obtain an absorbance of 0.700 at 734 nm. For quantification, 30 µL of extract were mixed with 570 µL of ABTS⁺ solution in 96-well microtiter plates and incubated at room temperature in the dark for 2 h. Then, the absorbance was read at 734 nm. TEAC was determined using a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve (0–100 mg/L) and calculated as micromolar of Trolox equivalents (µM TE) per gram of dry residue as follows:

\[
\text{Trolox equivalent antioxidant capacity (µM TE/g dw) = } \frac{\text{TEs} \times (V_s/M_s)}{\text{residue}}
\]

where TE is the antioxidant activity of each sample expressed in Trolox equivalents (µM), Vₛ is the sample volume (mL), and Mₛ is sample mass (g).

2.2.7. DPPH Free Radical-Scavenging Capacity

This method is based on the antioxidant scavenging of DPPH [16]. The reaction was carried out in 96-wells microplates and consisted of 30 µL of extracts and 1200 µL of methanolic solution of DPPH (6 × 10⁻⁵ M). The mixture was left for 40 min in the dark, and the reduction of DPPH was determined by measuring the absorption at 515 nm. Radical scavenging capacity (RSC) was determined from a Trolox calibration curve (0–1000 µM/L) and expressed in µM of Trolox equivalents (TE) per g dry extract weight (µM TE/g dw).

2.2.8. Antimicrobial Activity

The following nine strains were evaluated: three yeasts, *Saccharomyces cerevisiae*, *Candida albicans*, and *Kodamaea ohmeri*; three gram-positive bacteria, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Bacillus cereus*; and three gram-negative bacteria, *Shigella sonnei*, *Serratia marcescens*, and *Escherichia coli*. Antibacterial activity was measured via an agar disk diffusion test using Petri dishes with a PDA medium for yeast and a nutrient agar for bacteria. Dishes were inoculated with 0.1 mL of fresh cultures, adjusted to 0.5 of Mcfarland standard with a physiological solution. Sterile filter paper disks soaked with the different extracts were placed on the surface of the agar, and the inhibition zone diameter was measured after 48 h at 30 °C for yeast and after 24 h at 37 °C for bacteria. Paper discs with a 70% (v/v) ethanol solution were used as a control [28].

The minimal inhibitory concentration (MIC) was tested with five extract concentrations (10, 15, 20, 25, and 30% v/v). Potato dextrose broth (PDB) and nutrient broth were inoculated with 0.1 mL of fresh yeast and bacteria cultures, respectively, and adjusted to 0.5 of Mcfarland standard with a physiological solution. After incubation at 30 °C for 48 h for yeast and at 37 °C for 24 h for bacteria, the optical density of the cultures at 600 nm (OD₆₀₀) was determined. 70% (v/v) ethanol was used as a positive control, and cultures without hydrolysates were used as negative controls [29].

2.2.9. HPLC Analysis of Extracts

Next, RP-HPLC analysis was performed using an ACQUITY Arc system (Waters, Guadalajara, Mexico), equipped with a photodiode array detector (PDA) and a CORTECS C18 column (4.6 × 150 mm, 2.7 µm), (Waters, Guadalajara, Mexico). The column temperature was set at 30 °C, and the mobile phases used were A, water with TFA 0.1% v/v, and B, acetonitrile with TFA 0.1% v/v. Gradient elution was performed with a flow of 0.7 mL/min...
as follows: 0–10 min, 10–18% of B; 10–18 min, 18–28% of B; 18–19 min, 28–40% of B; 19–21 min, 40% of B; 21–23 min, 40–10% of B; 23–25 min, 10% of B. 10 µL of sample were injected and components were observed at 230, 280, 310, 360, and 520 nm. Catechin, epicatechin, apigenin, quercetin, rutin, ferulic acid, caffeic acid, gallic acid, 4-hydroxybenzoic acid, p-coumaric acid, cyanidin 3-glucoside, petunidin 3-glucoside, malvidin 3-glucoside, and delphinidin 3-glucoside pure standards were used for peak identification.

2.2.10. Statistical Analysis
The amount of phenolic content, flavonoids, anthocyanins, antioxidant activity, total sugars, and reducing sugars were measured in quadruplicate, and statistically significant differences between extracts were detected with ANOVA and the a posteriori Tukey’s HSD test using the software STATGRAPHICS Centurion XVI v.16.1.00. Significant differences were established at $p < 0.05$.

3. Results and Discussion

3.1. Agro-Industrial Residues Characterization
Each agro-industrial waste has characteristics that will affect its properties and potential applications. The chemical characteristics of the residues are presented in Table 1. The BAB was obtained dried, while BSG was dried before characterization. Blackberries and raspberries were characterized as collected, since drying negatively affected the recovery of carbohydrates and antioxidants (data not shown). The chemical composition of BAB is within the range of other profiles previously reported [19,30], and only the ash content was higher in the sample used in this study. Regarding BSG, the carbohydrate content is consistent with previous reports [4,31]. However, this concentration is higher than in several reports [18,32], since it is directly affected by the fermentation process used in the artisanal brewery.

Table 1. Chemical composition of agro-industrial wastes used.

| Component (%) | Blue Agave Bagasse (BAB) | Brewer’s Spent Grain (BSG) | Blackberry (BB) | Raspberry (RB) |
|---------------|--------------------------|--------------------------|-----------------|----------------|
| Moisture      |                          |                          |                 |                |
| Fresh †       | 9.05                     | 72.70                    | 79.26           | 88.16          |
| Dried †       | NM                       | 4.41                     | 2.7             | 6.85           |
| Ash           | 12.83                    | 2.05                     | 0.6             | 0.42           |
| Fats (ether extract) | 0.13                  | 5.46                     | 0.51            | 0.32           |
| Protein (N × 6.25) | 0.63                  | 18.56                    | 2.01            | 1.39           |
| Total sugars  | 71.29                    | 62.30                    | 17.67           | 9.71           |
| Reducing sugars | 51.70                | 33.44                    | 8.71            | 4.66           |
| Soluble lignin | 8.89                    | 3.03                     | ND              | ND             |

Values represent means of two measurements with standard deviations < 5%. † Moisture content at collection. ‡ Moisture content after drying. NM: Not measured; ND: Not detected.

The BB had a moisture content of 79.2%, comparable to the one reported by Muir et al. [33], but lower than most reports where the moisture content is between 87–93% [22,34,35]. These differences can be associated with the varieties, time of harvest, ripening, and maturity of the berries, in addition to each cultivar’s soil and weather conditions. This lower moisture content also explains the higher carbohydrate concentration found in the BB, which has been reported as being between 3% and 11% for fresh BB [22,34,35]. Reducing sugar concentration is similar to findings reported by Cunha et al. [36], but with a much lower moisture content (58%). In the case of RB, moisture, protein, fats, and carbohydrate contents are similar to a previous characterization of fresh RB [22].

3.2. Hydrolysates and Extracts Characterization
The extraction methods were selected since they can be considered environmentally friendly.
3.2.1. Sugars Content

The type of residue and extraction method influence the recovery of the compounds of interest. Total and reducing sugars of the hydrolysates and extracts are presented in Table 2. All residues presented their highest concentration of total sugars with CE hydrolysis, with over 60 g/L for BAB and BSG, 147 g/L for BB, and 47 g/L for RB. The highest concentration of reducing sugars for BAB and BSG was obtained with the CE method, while for BB and RB the EOH and CE methods gave the highest concentrations, respectively.

Table 2. Total and reducing sugars content in the aqueous and hydroalcoholic extracts, and chemical and enzymatic and enzymatic hydrolysates, of BAB, BSG, BB, and RB.

| Extract                | BAB     | BSG     | BB      | RB       |
|------------------------|---------|---------|---------|----------|
| Total sugars (g/L)     |         |         |         |          |
| Aqueous                | 2.95 ± 0.97 a | 26.09 ± 2.97 a | 46.35 ± 3.23 a | 43.32 ± 2.01 b |
| Enzymatic              | 19.85 ± 2.48 b | 33.33 ± 2.6 b   | 55.11 ± 6.56 a  | 44.28 ± 1.17 b  |
| Chemical and Enzymatic | 65.75 ± 3.17 c | 60.99 ± 2.58 c  | 147.44 ± 7.77 c | 47.14 ± 1.86 c  |
| Hydroalcoholic         | 1.53 ± 0.01 a | 32.07 ± 0.86 b  | 67.89 ± 0.54 b  | 22.05 ± 0.82 a  |

| Reducing sugars (g/L)  |         |         |         |          |
| Aqueous                | 0.68 ± 0.05 a | 24.94 ± 2.18 b | 26.09 ± 0.27 a | 15.16 ± 0.84 a |
| Enzymatic              | 14.01 ± 0.56 b | 27.28 ± 0.27 b | 38.57 ± 0.85 b | 15.79 ± 0.27 a |
| Chemical and Enzymatic | 51.51 ± 2.97 c | 33.46 ± 0.36 c | 39.75 ± 2.62 b | 22.34 ± 0.20 c |
| Hydroalcoholic         | 0.75 ± 0.01 a | 17.89 ± 0.79 a | 48.79 ± 0.39 c | 17.80 ± 0.77 b |

Superscript letters in the same column represent significant differences between extraction methods (p < 0.05) according to Tukey’s HSD test.

The concentration of reducing sugars extracted from BAB increased 20 and 75 times when using E and CE over AQ extraction, which was expected due to the nature of the residue. Since BAB is a low-cost substrate, hydrolysates of this residue have been used to produce polyhydroxybutyrate [19], ethanol [14], hydrogen [37–39], methane [20,37,38], lipids, and organic acids [12]. The BAB extract obtained with the CE method had 65.7 g/L and 51.5 g/L of total and reducing sugars, respectively. These results are higher than previous reports using acid hydrolysis (25.5 g/L TS and 20.6 g/L RS) [19], acid and enzymatic hydrolysis (17.3 g/L TS and 8.9 g/L TS) [37], enzymatic hydrolysis (12.6–17.5 g/L TS and 9–14 g/L RS) [39], and steam explosion (21.5 g/L TS) [20].

Brewer’s spent grain has been used to produce yeast biomass [4,10], enzymes [4,9], single-cell proteins [11], single-cell lipids [13], and carotenoids [10]. The highest concentration of total sugars for BSG was obtained with the CE treatment (60.9 g/L), with a reducing sugars concentration of 33.4 g/L. These results agree with the concentrations previously reported for acid pretreatments of BSG [40]. We have previously reported an aqueous extraction for BSG, obtaining 54 g/L of total sugars, although this was obtained using a 40% (w/v) residue concentration, 4 times higher than the conditions used in this study [4]. Enzymatic hydrolysis of BSG pretreated with microwave or organosolv generated hydrolysates with 51.4 g/L and 71.6 g/L of reducing sugars, respectively [13].

BB and RB production and consumption have increased in Jalisco, Mexico. Therefore, there is a great interest in the revalorization of those berries that do not meet commercial criteria. With both berries, the recovery of total sugars was maximized using the CE method. However, this increment was not proportional to the concentration of reducing sugars.

3.2.2. Phenolic Content

Phenolic content was quantified using FCR. This technique, despite its capacity to react with other non-phenolic compounds, such as vitamins and Maillard reaction products, is widespread due to its simplicity and reproducibility, and it is considered a routine technique for the quantification of phenolic compounds [41,42]. Figure 1 shows the total phenolic
content obtained in the different treatments. Results show that E, CE, and EOH extractions increased the recovery of phenolic compounds for all the samples when compared with the AQ extraction. An exception was observed in RB with E hydrolysis, where the recovery was lower. The highest phenolic content for BAB and BSG were obtained using the CE hydrolysis, reaching 11.32, and 9.89 mgGAE/g_residue, respectively.

![Figure 1. Total phenolic content in the BAB, BSG, BB, and RB extracts obtained with four treatments. For each residue, superscript letters indicate significant statistical differences between treatments (Tukey’s HSD test: p < 0.05).](image)

As previously mentioned, BAB hydrolysates provide the carbohydrates required to produce high added-value compounds but, unfortunately, during BAB hydrolysis, phenolic compounds that inhibit the fermentation processes are produced [14]. Regarding the phenolic compounds of BAB hydrolysates, significant difference was found between CE (11.30 mgGAE/g_residue, equivalent to 1.13 gGAE/L) and the rest of the treatments. This value is within range of those found in previous investigations, with concentrations of between 0.3 and 1.7 g/L [19,20,37], making the CE hydrolysates an excellent alternative to produce high added-value components from BAB through fermentation.

BSG is of interest for the extraction of high added-value products, such as phenolic compounds [27,40]. In the case of BSG, significant difference was observed between CE (9.89 mgGAE/g_residue equivalent to 989.58 µg/mL) and the other extractions methods used. The total phenolic content obtained with CE methods is more than 8.3 times higher than the content reported using acid pretreated BSG [40], and between eight and seventy times higher than extraction from untreated BSG [43,44], but lower than the one reported using microwave-assisted extraction [27].

Results show that for BB and RB, the ETOH treatment increased the recovery of phenolic compounds 4.0 and 2.2 times, respectively, when compared with the aqueous method. Phenolic content recovered from the BB after ETOH extraction (32.21 mg GAE/g_residue) is within range of the values in the literature, being higher than the one reported for BB seeds after Bligh–Dyer extraction [45], solvent extraction of lyophilized fruits [46–49], BB buds extracts [50], and aqueous extraction of freeze-dried fruits [51]. However, the values were lower than the total phenolics recovered from lyophilized BB, pressurized and ultrasound-assisted extraction of BB powder [21], aqueous and methanolic extracts of fresh fruits [52], sequential solvent extraction of mature fruits [22], and solvent and aqueous extraction of frozen fruits [35,36].

In the case of RB, total phenolic content after ETOH extraction treatment reached over 21.60 mg GAE/g_residue. This value is higher than the one reported for dried fruits after methanol-water extraction [48,53], aqueous extraction of freeze-dried fruits [51], RB
juice [54], RB buds extracts [50], and methanolic extract of lyophilized fresh pomace [49]. However, it is lower than aqueous and methanolic extracts of fresh fruits [32], dry extracts after solvent extraction [28], and sequential solvent extraction of mature fruits [22]. The concentration of secondary metabolites in RB change as a response to stress factors, such as light intensity and temperature [54]. The extraction method, in addition to fruits varieties, time of harvest, ripening, maturity state of the berries, soil, and weather conditions of the cultivar, highly affect the characteristics of the extracts and the differences found in the hydrolysates and extracts. It is also worth mentioning that a high phenolic concentration does not always translate into high antioxidant activity, since the quality of the compounds can be affected in the extraction process. Therefore, this activity will be discussed later.

3.2.3. Flavonoid Content

Flavonoid content is presented in Figure 2. For BAB and BSG, all treatments increased the flavonoid content compared with the AQ extraction, with the CE hydrolysates having ones with the highest concentrations of 10.0 and 5.2 mg RE/g residue, respectively. The flavonoid content of BAB CE hydrolysate is within the range of the concentration reported for other agave species [55,56]. Regarding BSG, CE hydrolysate flavonoid content was higher than the one reported after chemical hydrolysis [40] and solvent extraction [44] of this residue. In the case of BB and RB, the presence of enzymes negatively affected the recovery of flavonoids, meaning that the highest concentration was obtained after ETOH extraction. For BB, the EOH extraction had a flavonoid concentration of between 2.8 and 3.8 times higher than the other extraction techniques evaluated. This concentration is higher than the one found after pressurized liquid extraction of freeze-dried BB [21], solvent extraction of mature fruits [22], aqueous extraction of freeze-dried fruits [51], and aqueous and methanolic extraction of fresh fruits [52]. It was also lower than aqueous extraction of frozen fruits [35]. In the case of RB, the highest concentration with EOH extraction was 15.22 mg RE/g residue. The flavonoid concentration of RB was higher than the one reported for this fruit after different extraction techniques [22,51,52,54].

![Figure 2. Total flavonoid content in the BAB, BSG, BB, and RB hydrolysates and extracts obtained with four treatments. For each residue, superscript letters indicate significant statistical differences between treatments (Tukey’s HSD test: p < 0.05).](image)

3.2.4. Anthocyanin Content

The concentration of anthocyanins in the different extracts is presented in Table 3. The highest anthocyanin content from all the evaluated extracts was obtained with BB when hydroalcoholic extraction (96.3 mg CGE/100g residue) was used, followed by the EOH
extraction from RB (74.3 mg CGE/100g residue). For BAB, the anthocyanin content increased 3.1 times when using E and EOH extraction compared with AQ extraction. Anthocyanin extraction using BSG showed the highest recovery with CE. For BAB and RB, the chemical pretreatment caused degradation of the anthocyanins, obtaining the lowest concentration of these compounds for both residues with this extraction technique. The concentration of BB anthocyanin is higher than the value reported by Oszmiański et al. [21], but lower than several values found in the literature [22,35,47,49,51,52], while RB anthocyanin concentration is within the range of the concentrations reported by Bobinaitė et al. [28], but lower than in most reports [22,49,51,52]. Anthocyanin content in both berries was highly affected by overmaturity.

Table 3. Total anthocyanin content after aqueous and hydroalcoholic extraction, chemical and enzymatic, and enzymatic hydrolysates of BAB, BSG, BB, and RB.

|                 | BAB     | BSG     | BB      | RB      |
|-----------------|---------|---------|---------|---------|
| Aqueous         | 0.07 ± 0.01 a | 0.08 ± 0.01 a | 1.01 ± 0.08 a | 41.49 ± 1.0 b |
| Enzymatic       | 0.22 ± 0.06 b | 0.22 ± 0.03 a | 0.80 ± 0.09 a | 37.12 ± 3.13 b |
| Chemical and Enzymatic | 0.06 ± 0.00 a | 1.39 ± 0.03 d | 2.09 ± 0.33 a | 0.04 ± 0.02 a |
| Hydroalcoholic  | 0.22 ± 0.01 b | 1.23 ± 0.14 c | 96.32 ± 5.42 b | 74.36 ± 3.67 c |

Superscript letters indicate significant statistical differences between treatments (Tukey’s HSD test: p < 0.05).

3.2.5. Identification of Phenolic Compounds

The different residues studied in this work show a diverse and complex mixture of phenolic compounds that is difficult to characterize. The phenolic compounds identified and quantified in this study through HPLC are presented in Table 4. In the case of BAB, the highest concentration of phenolic compounds was observed for the CE treatment, which agrees with the data obtained after total phenolic quantification. The identified components with the highest concentrations after CE treatment of BAB in the hydrolysate were gallic acid (110.5 µg/g residue) and catechin (83.8 µg/g residue). In the case of BSG, the highest concentration of phenolic compounds was also found after CE treatment. However, in this case, rutin (229.6 µg/g residue), 4-hydroxybenzoic acid (118.0 µg/g residue), p-coumaric acid (59.7 µg/g residue), and gallic acid (40.5 µg/mL), were the identified components with the highest concentrations. In the case of berries, as observed in the previous sections, extraction of phenolic compounds was favored using the hydroalcoholic treatment, as the anthocyanins were the compounds with highest concentrations. A cyanidin 3-glucoside concentration in both ethanolic extracts of 4894.8 µg/g residue for BB and 658.8 µg/g residue for RB was obtained. In the case of BB cyanidin 3-glucoside was the major anthocyanin. However, for RB another compound with a higher peak area was separated, but not identified due to the lack of standards. According to the literature, cyanidin 3-sophoroside and cyanidin 3-glucoside are the major anthocyanins in RB [49,57]. In the ETOH extract of BB, catechin, epicatechin, rutin, caffeic acid, 4-hydroxybenzoic acid, p-coumaric acid, and petunidin 3-glucoside were also quantified. RB ETOH extract also presented catechin, caffeic acid, 4-hydroxybenzoic acid, p-coumaric acid, and petunidin 3-glucoside. All the phenolic compounds identified in this work agree with the information found in the literature. Unfortunately, despite the good resolution of the HPLC method used, several compounds were not identified, so only a partial profile can be found in Table 4.
Table 4. Major phenolic compounds identified for the hydrolysates and extracts of the four different residues studied.

|       | Catechin | Epicatechin | Rutin | Ferulic Acid | Caffeic Acid | Gallic Acid | 4-HBA | p-Coumaric Acid | C3G | P3G | Kaempferol |
|-------|----------|-------------|-------|--------------|--------------|-------------|-------|-----------------|-----|-----|------------|
|       |          |             |       |              |              |             |       |                 |     |     |            |
| BAB   |          |             |       |              |              |             |       |                 |     |     |            |
| AQ    | 17.6 ± 2.9 | N.D.        | N.D.  | 4.8 ± 0.3    | 3.1 ± 0.1    | 7.7 ± 0.1   | 10.8 ± 1.8 | N.D.            | N.D. | N.D. | 4.8 ± 0.3   |
| E     | 21.2 ± 0.5 | N.D.        | N.D.  | 7.1 ± 0.3    | 3.2 ± 0.3    | 8.5 ± 0.1   | 10.9 ± 0.1 | N.D.            | N.D. | N.D. | 3.3 ± 0.1   |
| CE    | 83.8 ± 7.3 | N.D.        | N.D.  | 7.4 ± 0.4    | 110.5 ± 7.7  | 9.2 ± 0.3   | 12.1 ± 0.2 | N.D.            | N.D. | N.D. | N.D.       |
| ETOH  | 19.5 ± 1.6 | N.D.        | N.D.  | 4.9 ± 0.6    | 54.6 ± 3.9   | 7.9 ± 0.1   | 10.2 ± 0.1 | N.D.            | N.D. | 4.1 ± 0.2 |
| BSG   |          |             |       |              |              |             |       |                 |     |     |            |
| AQ    | 8.8 ± 0.2  | N.D.        | N.D.  | 4.0 ± 0.1    | 3.0 ± 0.6    | 7.3 ± 0.1   | 10.9 ± 0.1 | N.D.            | N.D. | 3.8 ± 0.2 |
| E     | 18.7 ± 3.7 | N.D.        | 1.5 ± 0.1 | 4.7 ± 0.2   | 2.8 ± 0.1    | 9.6 ± 0.1   | 30.6 ± 3.5 | N.D.            | N.D. | 3.2 ± 0.2 |
| CE    | 9.9 ± 1.1  | 229.6 ± 16.5 | N.D.  | 10.5 ± 1.0   | 40.5 ± 3.8   | 118.0 ± 18.5 | 59.7 ± 6.9 | N.D.            | N.D. | 3.6 ± 0.2 |
| ETOH  | 14.2 ± 3.5 | N.D.        | N.D.  | 3.8 ± 0.2    | 15.4 ± 1.5   | 7.9 ± 0.1   | 14.1 ± 1.9 | N.D.            | N.D. | 3.9 ± 0.1 |
| BB    |          |             |       |              |              |             |       |                 |     |     |            |
| AQ    | 12.9 ± 2.7 | N.D.        | N.D.  | 33.9 ± 0.1   | 12.2 ± 1.5   | 73.0 ± 0.1  | 95.1 ± 2.1 | N.D.            | N.D. | N.D. | N.D.       |
| E     | 8.0 ± 1.4  | N.D.        | N.D.  | 33.9 ± 0.1   | N.D.         | 74.1 ± 1.1  | 100.1 ± 0.2 | N.D.            | N.D. | N.D. | N.D.       |
| CE    | 26.1 ± 0.9 | N.D.        | N.D.  | 40.2 ± 1.3   | 293.8 ± 22.6 | 77.3 ± 1.2  | 95.1 ± 0.6 | N.D.            | N.D. | N.D. | N.D.       |
| ETOH  | 48.9 ± 3.7 | 1171.1 ± 58.3 | 71.3 ± 15.0 | 73.7 ± 5.8 | 162.4 ± 19.9 | 223.3 ± 19.1 | 4894.8 ± 334.2 | 322.7 ± 16.1 | N.D. | N.D. | N.D.       |
| RB    |          |             |       |              |              |             |       |                 |     |     |            |
| AQ    | 35.0 ± 0.9 | N.D.        | N.D.  | 3.9 ± 0.3    | 1.9 ± 0.6    | 77.2 ± 4.4  | 95.9 ± 3.1 | 318.6 ± 12.1   | 300.2 ± 7.6 | N.D. |
| E     | 35.1 ± 1.3 | N.D.        | N.D.  | 42.5 ± 1.8   | 5.3 ± 0.2    | 81.0 ± 1.4  | 97.0 ± 1.6 | 105.4 ± 23.2   | 312.5 ± 9.5 | N.D. |
| CE    | 38.9 ± 3.3 | N.D.        | N.D.  | 52.2 ± 5.4   | 47.4 ± 1.1   | 138.5 ± 6.4 | 77.2 ± 0.2 | 97.6 ± 0.2    | N.D. | N.D. | N.D.       |
| ETOH  | 132.4 ± 11.0 | N.D.     | N.D.  | 67.4 ± 0.3   | N.D.         | 136.9 ± 0.2 | 197.3 ± 11.0 | 658.8 ± 60.2   | 515.9 ± 41.2 | N.D. |

Values represent means of two measurements with standard deviations <5%. 4-HBA: 4-hydroxybenzoic acid; C3G: cyanidin 3-glucoside; P3G: petunidin 3-glucoside; N.D., not detected.
3.2.6. Antioxidant Activity

Antioxidant activity was measured using a ABTS and DPPH radical scavenging capacity (Table 5). The results showed that the antioxidant activity depended on the treatment, condition of the extraction, and the composition of the residue, in agreement with other studies [43]. Results showed that the best treatments to release bioactive compounds were CE for all residues when the activity was measured with ABTS. When the antioxidant activity was measured with DPPH, the best extraction technique was CE for BAB and BSG, and EOH for BB and RB. According to the techniques used, results may present variations.

Table 5. Antioxidant capacity of aqueous and hydroalcoholic extracts, chemical and enzymatic, and enzymatic hydrolysates of BAB, BSG, BB and RB using ABTS and DPPH.

|           | BAB                | BSG                | BB                  | RB                  |
|-----------|--------------------|--------------------|---------------------|---------------------|
| **ABTS (µM TE/g)** |                   |                    |                     |                     |
| Aqueous   | 17.83 ± 1.49 a     | 10.89 ± 0.18 a     | 34.28 ± 1.35 b     | 45.99 ± 2.90 a     |
| Enzymatic | 20.96 ± 4.75 a     | 14.04 ± 0.79 a     | 23.62 ± 0.66 a     | 39.72 ± 1.45 a     |
| Chemical and Enzymatic | 29.81 ± 2.31 b | 29.36 ± 5.10 b     | 181.67 ± 5.54 d    | 146.59 ± 10.15 c  |
| Hydroalcoholic | 19.67 ± 1.28 a   | 11.03 ± 0.77 a     | 82.47 ± 3.80 c     | 77.96 ± 3.096 b   |
| **DPPH (µM TE/g)** |                   |                    |                     |                     |
| Aqueous   | 3.75 ± 0.34 a      | 1.57 ± 0.06 a      | 7.81 ± 0.19 b      | 8.55 ±0.09 c      |
| Enzymatic | 4.10 ± 0.96 a      | 3.72 ± 0.22 c      | 6.36 ± 0.27 a      | 7.63 ± 0.28 b     |
| Chemical and Enzymatic | 5.35 ±0.17 b | 4.99 ± 0.57 d      | 8.24 ± 0.17 b      | 4.74 ± 0.29 a     |
| Hydroalcoholic | 4.04 ± 0.19 a    | 2.38 ± 0.25 b      | 16.93 ±0.37 c      | 15.27 ± 0.11 d   |

Superscript letters indicate significant statistical differences between treatments (Tukey’s HSD test: p < 0.05).

According to the results found in this study for BAB, the highest values of antioxidant capacity were obtained with the CE treatment (29.81 ± 2.31 µM TE/g) and (5.35 ± 0.17 µM TE/g) using ABTS and DPPH, respectively. There are limited studies on the antioxidant capacity of agave and its derivatives. However, the values found in this work are higher than the ones published by Ahumada-Santos et al. [58], who reported 9.86 µM TE/g (ABTS assay) in a methanolic extract of plant leaves. Agave leaves had no treatment before the methanolic extraction, explaining the low activity found. Since BAB was recovered after physical and thermal treatments used in juice extraction for tequila production, these processes can favor the release of molecules with biological activity.

Regarding BSG, the highest value was found in the CE hydrolysate with ABTS and DPPH were 29.36 ± 5.19 and 4.99 ± 0.57 µM TE/g respectively, and these values were significantly different to the other treatments. The antioxidant activity found in this study is higher than those published in the literature. Socaci et al. [44] reported antioxidant activity of hydroalcoholic (60% ethanol) and AQ extracts of 1.35 µM TE/100 g and 1.09 µM TE/100 g respectively, and Bonifácio-Lopes et al. [43] referred values of 0.28 and 0.77 mg acid ascorbic/g BSG in AQ and EOH extracts (60% ethanol). Under similar extraction conditions (AQ and EOH), the values found in this study were 0.32 and 0.59 mg TE/g with DPPH assay. Nevertheless, the antioxidant activity of the BSG depends on method and extraction conditions, in addition to the type of malt and fermentation used [27,43].

For the BB, the highest value of antioxidant activity was obtained after CE hydrolysis and EOH extraction with ABTS and DPPH methodologies, respectively. According to statistical analysis, the value obtained with treatment CE and EOH is significantly different from the rest of the treatments. The extraction method impacts the quality of the product obtained and its biological activity. For example, using ultrasound and pressurized liquid extraction, BB extracts presented antioxidant activity values between 50 and 90 µM TE/g [21], values lower than those found in this study. In the chemical enzymatic hydrolysate, the value found was 181.67 µM TE/g with ABTS, which is 3.6 and 2 times higher than the values reported by Oszmianański et al. [21].
In the case of RB, the extracts with the best antioxidant activity were obtained with CE and EOH, for ABTS (146.59 ± 10.15 µm TE/g) and DPPH (15.27 ± 0.11 µm TE/g), respectively, which was significantly different from the rest of the values. The antioxidant activity reported in the literature for extracts obtained with organic solvents and with enzymes were 285.5 and 342 µM TE/g, respectively [23]. On the other hand, Bobinaitė et al. [28] reported the antioxidant activity of methanolic extracts from different raspberry cultivars, obtaining values of 459.8, 299.8, and 334.2 µM TE/g. The results obtained in our study are lower than the mentioned, however, the antioxidant capacity depends on cultivar, maturity level, and type and conditions of the extraction method.

Antioxidant activity has been related to extracts’ phenolic content [52,58,59]. In this study, the extracts with the highest antioxidant activity in BAB and BSG presented the highest phenolic, flavonoids and anthocyanins concentration. For the extracts of BB and RB the highest antioxidant activity (with DPPH) coincides with high concentration of phenolic, flavonoids and anthocyanins.

3.2.7. Antimicrobial Activity

A screening incorporating concentrated hydrolysates and extracts to the growth media (data not shown) revealed higher antimicrobial activity for CE hydrolysate of BAB and E hydrolysates of BSG, BB, and RB. These hydrolysates were selected for the agar diffusion test and MIC. The inhibition halo generated by the different extracts is shown in Table 6. In the case of B. cereus, S. saprophyticus, S. sonnei, and C. albicans, the most significant effect was found with the BSG hydrolysate. S. aureus and E. coli, showed higher sensitivity with the raspberry E hydrolysate. E hydrolysate of BB generated inhibition halos in seven of the nine strains evaluated, while E hydrolysate of RB inhibited seven strains and CE hydrolysate of BAB only four. Under the conditions studied, no inhibition halo was detected for K. ohmeri or S. cerevisiae with any of the extracts; however, it is possible that the excess sugar present in the hydrolysates stimulated the growth of these yeasts.

Table 6. Antimicrobial activity (inhibition halo) in the aqueous and hydroalcoholic extracts and Chemical and enzymatic and enzymatic hydrolysates of BAB, BSG, BB and RB.

| Strain       | BAB Inhibition Halo (mm) | BSG Inhibition Halo (mm) | BB Inhibition Halo (mm) | RB Inhibition Halo (mm) | Control Inhibition Halo (mm) |
|--------------|--------------------------|--------------------------|-------------------------|-------------------------|-----------------------------|
| S. cerevisiae| 0                        | 0                        | 0                       | 0                       | 4.46 ± 0.09                 |
| C. albicans  | 0                        | 1.06 ± 0.04              | 0.91 ± 0.05             | 0.65 ± 0.03             | 1.30 ± 0.02                 |
| K. ohmeri    | 0                        | 0                        | 0                       | 0                       | 0.77 ± 0.01                 |
| S. aureus    | 0.70 ± 0.02              | 0.93 ± 0.02              | 0.71 ± 0.03             | 0.69 ± 0.01             | 1.17 ± 0.06                 |
| S. saprophyticus | 1.88 ± 0.06              | 1.35 ± 0.03              | 0                       | 0                       | 0.63 ± 0.05                 |
| B. cereus    | 0.31 ± 0.03              | 0.34 ± 0.02              | 0.35 ± 0.02             | 0.1 ± 0.01              | 0.7 ± 0.04                  |
| S. sonnei    | 0.5 ± 0.02               | 0.5 ± 0.02               | 0.5 ± 0.02              | 0.5 ± 0.02              | 0.75 ± 0.06                 |
| S. marcescens| 0.29 ± 0.02              | 0.31 ± 0.02              | 0.54 ± 0.02             | 0.51 ± 0.04             | 1.27 ± 0.01                 |
| E. coli      | 0.48 ± 0.02              | 0.58 ± 0.03              | 0.67 ± 0.04             | 0.77 ± 0.01             | 1.27 ± 0.01                 |

The BAB sample showed increased antimicrobial activity against gram-negative bacteria, similar to the behavior observed in previous studies [58], but lower than the control, in addition to showing no effect against the yeasts evaluated. Antibacterial activity of the BSG extract was observed with all the bacteria used in this study and the yeast C. albicans. Socaci et al. [44] published similar results with methanol, ethanol, acetone, hexane, ethyl acetate, and water extracts. Bonifácio-Lopes et al. [43] also reported antimicrobial activity of a BSG hydroalcoholic extract against E. coli, B. cereus, and Listeria monocytogenes. The BB enzymatic hydrolysate did not show antimicrobial activity against S. cerevisiae, and K. ohmeri, but had a good response with the other microorganisms used in this work. Krzepilko et al. [50] found antimicrobial activity in ethanolic extracts of two cultivars of BB against Enterobacter aerogenes, Enterococcus faecalis, and S. aureus. In another study, results from the antimicrobial activity against gram-positive and gram-negative bacteria, E. coli, E.
faecalis, and S. aureus, and some yeasts, C. albicans and Candida parapsilosis, of BB juice from three different cultivars [60], agree with our findings. RB extract showed antimicrobial activity versus C. albicans, S. aureus, S. saprophyticus, S. sonnei, S marcescens, and E. coli (yeast, gram-positive, and gram-negative bacteria).

Minimum inhibitory concentration (MIC) results show inhibition for BSG, BB, and RB hydrolysates (Figure 3), while BAB CE hydrolysate had no antimicrobial effect. The BSG extract showed antimicrobial activity against gram-positive and gram-negative bacteria, but not against yeast. The 10% (v/v) hydrolysate reduced the OD of the gram-negative bacteria, but the effect is more evident with 15% of hydrolysate. The OD of the cultures decreased from 0.2 to 0.1 for S. marcescens and S. sonnei, while for E. coli, it decreased from 0.3 to 0.1 with 15% of hydrolysate. A similar behavior was observed for the gram-positive bacteria, S. saprophyticus and B. cereus, whose OD decreased with 10% of the crude extract, reaching the highest growth inhibition with 15%. On the other hand, S. aureus showed a different behavior. The 10% crude extract stimulated its growth, and growth inhibition was observed starting from 15%, reaching 0.1 OD with the maximum concentration used in this test (30%). In this study, the BSG hydrolysate appears to be effective against both groups of bacteria. Although the use of organic solvents to extract bioactive compounds is widespread due to their effectiveness, this work looked to validate environmentally friendly alternatives, trying to preserve or even improve the effectiveness reported with organic solvents. The BSG hydrolysate obtained in this work showed antimicrobial activity similar to that reported by Socaci et al. [44].

Figure 3. Inhibition effect at five extract concentrations. (a) BSG, gram-positive strains, (b) BSG, gram-negative strains, (c) BB, gram-positive strains, (d) BB, gram-negative strains, (e) RB, gram-positive strains, (f) RB, gram-negative strains.
(f) RB, gram-negative strains. (♦) *S. saprophyticus*, (●) *S. aureus*, (□) *B. cereus*, (○) *E. coli*, (☐) *S. sonnei*, and (■) *S. marcescens*.

The BB hydrolysate did not show antimicrobial activity against gram-positive bacteria. However, the antimicrobial effect of the BB extract was evident against *S. sonnei* and *S. marcescens* where the OD of the cultures decreased with concentrations of 10% and 20%, respectively. The antimicrobial activity of BB juice was reported against gram-positive, gram-negative bacteria, and yeasts [60]. Furthermore, Junior et al. [45] reported the antimicrobial effect of BB extracts obtained with organic solvents against *E. coli* and *S. aureus*, although this behavior that was not observed in this work. However, the extraction method and part of the plant used influence the biological activity of the extracts [45,48], and would explain the differences between the reported studies.

The RB extract showed an evident antimicrobial activity against gram-positive and gram-negative bacteria. The concentration at which a decrease in OD was observed for almost all bacteria was 10%. Only with *S. aureus* and *E. coli* was no inhibition observed. These results coincide with what was reported by Bobinaitè et al. [28].

4. Conclusions

Under the conditions evaluated, it was possible to extract sugars and compounds with antioxidant and antimicrobial activity from the four chosen residues. In addition, it was observed that the extraction process and the nature of the materials evaluated directly influenced the quality of the extracts obtained. For the recovery of total and reducing sugars, the best method for the four residues evaluated was CE hydrolysis, so these hydrolysates can be used as raw material to obtain value-added products through fermentation. In general, CE hydrolysis was the best treatment to recover phenolic, flavonoid, and anthocyanin compounds and hydrolysates with antioxidant activity from BAB and BSG, and from lignocellulosic residues. The best treatment to recover phenolic, flavonoid, and anthocyanin compounds and hydrolysates with antioxidant activity from BB and RB was EOH. The hydrolysate with the best antimicrobial activity was RB, follow by BB and BSG. The revalorization of these four residues to obtain molecules with high added-value is an environmentally and economically attractive option, since these molecules can be used in the pharmaceutical, food, and cosmetics industries.

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