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Abstract: The recovery of antioxidants from complex winery and grapevine by-products into Vitis vinifera must offers new opportunities for wine grapes by the development of a new, enriched fruit juice. However, this demands the search for new valorization methods to get hold of additional antioxidant compounds. The objective of this study was to find a novel functionality for grape pomace, grapevine leaves, and canes by its reuse as a functional matrix for the extraction of antioxidants into grape must. After thermomaceration, 22 polyphenols were identified by high performance liquid chromatography and mass spectrometry. Grape pomace was a good source of anthocyanins (malvidin-3-glucoside), while flavonols (quercetin-3-hexoside) and phenolic acids (caftaric acid) were the main phenolic compounds in leaf extracts. Catechin dimer was the only polyphenol compound present in all of the matrices. Enriched grape juice comprised by 40:20:40 (v/v/v) of pomace, leaf, and cane extracts, yielded an oxygen radical absorbance capacity of pirogallol red and fluorescein ratio of 0.70, indicating that the reactivity of antioxidants present in enriched grape juice was at least as efficient as other polyphenol-rich beverages. Thus, pomace, leaves and canes supply additional polyphenols to grape must that results into a beverage with promissory antioxidant activity and potential health benefits.

Keywords: grape juice; thermomaceration; antioxidants; polyphenols; Vitis vinifera

1. Introduction

Since the observation of a lower mortality rate of coronary heart disease in France when compared to Northern European countries, known as the “French paradox” [1], a number of studies showed the health-promoting effects of phenolic compounds that are present in grapes and grape-derived products, including pure grape juice [2–5]. Consequently, there is a steady global rise of grape juice production over the last thirty years to fulfil a growing demand for pure grape juice by health-conscious consumers. However, even if grape juice meets all health requirements, flavor and other product attributes are critical for consumer acceptance. Thus, the increasing demand for healthy, sensory attractive fruit juices by more demanding, better-educated consumers requires a continuous need for the development of new juice products.
After comparing 13 commercially available fruit juices and juice drinks, purple grape juice contained the highest levels of polyphenols and antioxidants [6]. Phenolic acids and flavan-3-ols were the predominant compounds in white grape juice, while the major groups of polyphenols that were found in purple and red grape juices comprised anthocyanins and flavan-3-ols [6–9]. In particular, several health benefits are associated with the consumption of purple grape juice, such as an improved endothelial function, protection against LDL cholesterol oxidation, decrease in LDL-HDL cholesterol ratio, inhibition of atherosclerosis, improved neurocognitive function, and improved antioxidant biomarkers in blood [2,10–12]. The main beneficial effects of purple grape juices may be due to their contents of flavonoids. In particular, procyanidin dimers, flavonols, and flavan-3-ols show high antioxidant capacity among other polyphenols [13]. Primarily, flavonols and proanthocyanins are associated with a marked decrease in platelet superoxide production and inhibition of platelet aggregation [14], while oligomeric procyanidins improve vascular health [15]. Moreover, anthocyanins, catechin, procyanidins, and E-resveratrol from grape skins and seeds show an inhibition of the growth of human cancer cells [16–18], while anthocyanins and E-resveratrol may also suppress inflammatory reactions [19].

Despite of high phenolic contents of grapes, the processing of grapes results in high amounts of by-products, whereby a major part of the phenolics remain within the grape pomace after processing. However, the content in bioactive phytochemicals that are detected in grape residues shows a strong variation due to different agro-climatic and recovery conditions. Concerning the importance of the up to nine million tons of vine and winery by-products produced globally every year from grape industrialization [20], these by-products could provide extra desirable ingredients for health-food applications improving juice quality [21,22]. Therefore, the aim of the present study was to find a novel functionality for grape pomace, grapevine leaves, and canes by its reuse as a functional matrix for the extraction of antioxidants into must of *Vitis vinifera* grapes. At the same time, it helps to add value to those minor red grape cultivars that are not destined to the production of fine wines.

### 2. Materials and Methods

#### 2.1. Reagents and Standards

Commercial standards of delphinidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-glucoside, quercetin-3-glucuronide, and chlorogenic acid were obtained from PhytoLab (Vestenbergsgreuth, Germany). Extrasynthese (Lyon, France) provided peonidin-3-glucoside. Commercial standards of quercetin-3-rutinoside, quercetin-3-glucoside, gallic acid, ferulic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Merck (Darmstadt, Germany) provided formic acid, acetonitrile, methanol and water (all HPLC grade), while neocuproine hemihydrate was obtained from Fluka (Buchs, Switzerland).

#### 2.2. Samples

Grapes, grapevine leaves and canes from *Vitis vinifera* L., cvs. Pais (PA; an ancient, red cultivar) and Lachryma Christi (LC; a Teinturier cultivar, commonly used for blending with pale red wines to intensify red color) were collected from two vineyards that were located in the Itata Valley, San Nicolas, Chile (36°33′ S–72°10′ W and 36°30′ S–72°05′ W, respectively) between March and June 2013. After destemming, grapes were crushed (PAS.0540, Bertuzzi, Brugherio—Milano, Italy) and pressed (D.64625, Willmes, Bensheim, Germany), prior to the collection of grape must and pomace (skins and seeds) and storage at −20 °C. After vintage, autumn leaves and canes were cut and stored at −20 °C until solid-liquid extraction.
2.3. Extraction Procedure

Aqueous extracts from unfrozen, ground (1–2 mm) pomace, leaves and canes were prepared by using grape must as a solvent. Thermomaceration was carried out by mixing 55 g of winery or grapevine by-product with 445 g of grape must in a 1-L double-wall glass vessel, stirred at 500 rpm (Barnstead Thermolyne, Super Nuova magnetic stirrer, Thermo Scientific, Ashville, NC, USA). Temperature was set at 60 °C using a Thermo/Haake DC10-K10 circulating heating bath (Haake, Karlsruhe, Germany), while the extraction vessel was duly covered by parafilm and aluminum foil to avoid solvent loss and light influence. The process time was fixed at 4, 6, and 8 h for cane, pomace, and leaf samples, respectively, in order to achieve a grape must with a maximum amount of polyphenols and antioxidants [23]. Then, enriched grape juice was prepared by mixing pomace, leaf and cane extracts as follows: after filtration using 20–25 µm nylon filter bags, pomace, leaf, and cane extracts were mixed in a ratio of 40:20:40 (v/v/v). After combining 250 mL of the País mixture to an equal amount of Lachryma Christi mixture, the resulting blend, called enriched grape juice, was bottled, heated (63 °C for 30 min), and stored at 20 °C until analysis.

2.4. Analytical Methods

2.4.1. Physicochemical Characterization

Standard enological parameters, such as pH, total acidity, soluble solids, free and total SO₂, and chromatic characteristics, were measured in must and enriched juice samples, according to the official OIV methods of analysis [24]. All of the measurements were done in triplicate.

2.4.2. Spectrophotometric Assays

Spectrophotometric analyses were performed using an Analytik Jena Specord 200 Plus spectrophotometer (Jena, Germany) set at the appropriate wavelength for each assay.

Total polyphenol content was measured by using the Folin-Ciocalteu colorimetric assay and the results were reported in mg/kg of gallic acid equivalents [25]. Monomeric anthocyanin content was analyzed using the pH-differential method, expressing the results in mg/kg of malvidin-3-glucoside equivalents [26]. Total flavonoid content was evaluated using a colorimetric assay with aluminum chloride using catechin as standard [27].

Additionally, total antioxidant capacities were evaluated by the ABTS (2,2′-azinobis(3-ethylbenothiazoline-6-sulfonic acid)) radical cation assay, CUPRAC (cupric reducing antioxidant capacity) method, ORAC (oxygen radical absorbance capacity)—fluorescein (FL), and ORAC—pirogallol red (PGR) assays, as described previously [23,28–30]. In all of the assays, Trolox was used as reference compound and results were expressed in terms of mmol Trolox equivalent antioxidant capacity per kg of sample. All of the measurements were done in triplicate.

2.4.3. Sample Preparation

Prior to HPLC separation, a solid-phase extraction using Oasis MCX cartridges (Waters, Milford, MA, USA) containing a mixture of reverse-phase and cation exchange materials allowed for the separation of anthocyanins from the remaining phenolic compounds to allow their determination without interference. Five milliliters of each must or extract sample were diluted with 5 mL of 0.1 M hydrochloric acid. This solution was passed through the 500 mg Oasis MCX cartridge previously conditioned with 5 mL of methanol and 5 mL of water. After rinsing with 5 mL of 0.1 M hydrochloric acid and 5 mL of water, the fraction containing flavonols, flavan-3-ols, and phenolic acid derivatives was eluted by passing 3 × 5 mL of methanol, while the anthocyanins remained in the solid phase. The anthocyanins were recovered by eluting 10 mL of 5% w/v ammonium hydroxide in methanol. Subsequently, the solvents of these fractions were removed by vacuum rotary evaporation and the residues dissolved in 5 mL of mobile phase used in HPLC separation.
2.4.4. HPLC-DAD-ESI-MS/MS

HPLC separation, identification, and quantification of specific phenolic compounds were carried out using a Shimadzu HPLC Nexera system (Kyoto, Japan). This equipment consists of a quaternary LC-30AD pump, DGU-20A degasser unit, CTO-20AC oven, SIL-30AC auto-sampler, CBM-20A controller system, and UV-Vis diode array spectrophotometer (model SPD-M20A), coupled in tandem with a QTrap LC/MS/MS 3200 Applied Biosystems MDS Sciex system (Foster City, CA, USA). The detector offers wide linearity (2.5 AU) and a noise level of $0.6 \times 10^{-5}$ AU for a wavelength from 190 to 700 nm. Instrument control and data collection were done using CLASS-VP DAD Shimadzu Chromatography Data System and Analyst software (version 1.5.2) for MS/MS analysis.

Anthocyanins were separated by HPLC using a C18 YMC 5 µm, 250 × 4.6 mm column with a C18 Nova-Pak 4 µm, 22 × 3.9 mm precolumn (Waters, Milford, MA, USA) with a flow rate of 0.3 mL/min at 30 °C. The injection volume was 50 µL. The mobile phase consisted of 0.1% v/v trifluoroacetic acid in water (A) and 100% acetonitrile (B). The gradient program was from 10% to 20% of solvent B in 15 min, followed by 6 min of stabilization, from 20% to 27% in 5 min, followed by 10 min stabilization, from 27% to 100% in 1 min, and from 100% to 10% in 1 min, followed by an isocratic step of 10 min at 10% B.

HPLC separation of flavonols, flavan-3-ols and phenolic acid derivatives was carried out using a Kinetex C18 column (core shell, 150 × 4.6 mm, 2.6 µm) with a SecurityGuard AJO-8768 C18 cartridge (Phenomenex, Torrance, CA, USA). The injection volume was 10 µL. A binary mobile phase of 0.1% v/v formic acid in water and acetonitrile was used at a flow rate of 0.5 mL/min. The mobile phase gradient ranged from 15% to 25% acetonitrile for 14 min, from 25% to 35% for 11 min, from 35% to 100% for 1 min, from 100% to 15% for 1 min, followed by a stabilization period of 10 min. The column temperature was set at 30 °C for flavan-3-ols and phenolic acid derivatives, and at 40 °C for flavonols.

The analyses of stilbenoids were carried out using a C18 Kromasil 5 µm, 250 × 4.6 mm column (Akzo Nobel, Bohus, Sweden) with a C18 Nova-Pak Waters 22 × 3.9 mm, 4 µm precolumn (Waters, Milford, MA, USA) at 30 °C, using a mobile phase gradient consisting of 0.1% v/v formic acid in water (solvent A) and acetonitrile (solvent B). The injection volume was 25 µL. The flow rate was 0.5 mL/min, and the gradient program was from 15% to 20% of solvent B in 5 min, 20% to 44.5% in 45 min, and 44.5% to 100% in 1 min, followed by an isocratic step of 9 min at 100% and stabilization for 5 min at 15% of B.

The identity of phenolic compounds was assigned by ESI-MS/MS setting the following parameters: negative ionization mode; collision energy, 5 V; ionization voltage, −4000 V; capillary temperature, 450 °C; nebulizer gas, 15 psi. For identification of anthocyanins, a positive ionization mode was used. The identity assignation of compounds was done by comparison of their retention time ($t_R$), UV-Vis spectra and mass (MS/MS) spectra with those of their respective commercially available standards. Quantification was performed using a DAD chromatogram extracted at 280 nm for flavan-3-ols, 306 nm for stilbenoids, 320 nm for phenolic acid derivatives, 360 nm for flavonols, and 518 nm for anthocyanins. For quantitative determinations, calibration curves were made with the commercial standards for flavan-3-ols, stilbenoids, phenolic acids, flavonols, and anthocyanins. Standard solutions spanning the concentration range from 1.0 to 80 mg/L were prepared by appropriate dilution of standard solutions in solvent A. The limits of detection and quantification were three and ten times the noise signal from the chromatograms of low standard concentration.

2.5. Sensory Analysis

Sensory analysis was performed using a tasting panel of twelve trained judges (six men and six women, aged 25–62 years, consisting of students and university employees) that compared sensory attributes of several mixtures of pomace, leaf, and cane extracts of both Pais and Lachryma Christi grapes. Panelists were seated in separate booths, each with appropriate lighting, ventilation, and free from noise and other distracting stimuli. Thirty-milliliter samples coded with a random three-digit number were presented at 20 °C to each panelist in blue color glass cups. Samples were
tested in three sessions over different days by preference ranking to evaluate flavor, taste, and tactile attributes, where the judges were presented with the samples and were instructed to indicate their preference for each sample on a hedonic scale (0–10) and to order them from least (0 score) to most preferred ones (10 score). Panelists had to eat some piece of cracker and rinse their mouth with water to reduce carry-over effects between sample evaluations. At the end of each session, the judges completed a questionnaire to give their perceptions of sensory attributes. The questionnaire included a list of descriptors of odor (fruity, herbal, flowery, woody, spicy), taste (sweetness, acidity, bitterness, salty), and tactile attributes (astringency, sandy).

2.6. Statistical Analysis

Analysis of variance was performed to assess statistically significant differences between data of grape juice samples at a confidence level of 95% using the Statgraphics Centurion XVII software, version 17.1.06 (Statpoint Technologies, Warrenton, VA, USA). Differences on the means were assessed by Duncan’s multiple range test at a significance level of \( p < 0.05 \), using the Fisher’s least significant difference procedure. Preference ranking data were analyzed using the Kruskal-Wallis test for non-homogeneous samples at \( p = 0.05 \).

3. Results and Discussion

3.1. Phenolic Composition

In the current study, 22 polyphenol compounds, including five anthocyanins, eight flavonols, six phenolic acids, and three flavan-3-ols, were identified by HPLC-DAD-ESI-MS/MS in grape musts and extracts (Table 1). An unknown compound from Lachryma Christi grapes being a hydroxycinnamic acid derivative according to its UV-spectra did not provide enough molecular information in order to establish its identity. Malvidin-3-glucoside, quercetin-3-hexoside, caftaric acid, and catechin dimers were the predominant compounds that were detected in these samples. Amongst polyphenols, catechin dimer was the only component detected both in PA and LC must. Moreover, this component was detected in all of the samples. A low number of phenolic acids and flavan-3-ols, and the absence of anthocyanins and flavonols are observed in PA must, in contrast to LC must, which was particularly rich in flavan-3-ols and anthocyanins. This agrees with the high levels of flavan-3-ols and anthocyanins reported for purple grape juice among 13 commercial fruit juices [6].

Pomace of Pais grapes is an additional source of a diversity of phenolic compounds, supplying substantial amounts of malvidin-3-glucoside, quercetin-3-hexoside, caftaric and coumaric acid, catechin, and epicatechin to grape must (Table 2). Pomace from Lachryma Christi grapes yielded an extract, which was highly fortified in anthocyanins with a maximum concentration of 1563 mg malvidin-3-glucoside/kg extract (Table 2).

Additionally, delphinidin-3-glucoside and petunidin-3-glucoside are localized typically in grape skins and seeds, but not in the pulp, being absent in must from Lachryma Christi grapes. Anthocyanins from grape skins include malvidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, amongst others [31,32]. However, grape seeds may also provide anthocyanins to the must that depends on process conditions, such as time, temperature, and solvent and ultrasound assistance to extraction [33]. Malvidin-acetyl-glucoside, detected in must and pomace extract from Lachryma Christi grapes seems to be involved in complex formation with other organic cofactors, which results in a product with a deep dark purple color. In Pinot noir and Sangiovese cultivars, the lack of acetylated anthocyanins caused a minimum level of co-pigmentation [34].

Furthermore, pomace of Lachryma Christi cultivar provided other phenolic compounds, such as myricetin, quercetin, and isorhamnetin derivatives, and hydroxybenzoic and hydroxycinnamic acid derivatives, lacking in grape must. Although most of the anthocyanins and flavan-3-ols (catechin, epicatechin, and procyanidin B2) are generally removed as skins and seeds during juice
processing [35–37], bioactive contents from grape pomace may still become available to fortify juice after applying thermomaceration.

Table 1. Chemical characterization (HPLC-DAD-ESI-MS/MS) of polyphenols in grape musts and extracts.

<table>
<thead>
<tr>
<th>Name</th>
<th>Iq (min)</th>
<th>[M-H] (m/z)</th>
<th>Product Ions (m/z)</th>
<th>λ_{max} (nm)</th>
<th>Detected in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>16.88</td>
<td>465</td>
<td>303</td>
<td>524, 277, 343</td>
<td>f</td>
</tr>
<tr>
<td>Petunidin-3-glucoside</td>
<td>20.23</td>
<td>479</td>
<td>317, 302</td>
<td>525, 277</td>
<td>f</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>22.54</td>
<td>463</td>
<td>301, 286</td>
<td>517, 279</td>
<td>e, f, g, h</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>23.36</td>
<td>493</td>
<td>331, 315, 287</td>
<td>527, 277, 346</td>
<td>b, e, f, g, h</td>
</tr>
<tr>
<td>Malvidin-acetyl-glucoside</td>
<td>34.30</td>
<td>535</td>
<td>331, 315, 287</td>
<td>529, 525</td>
<td>e, f</td>
</tr>
<tr>
<td>Flavonols</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myricetin-3-hexoside</td>
<td>7.04</td>
<td>493</td>
<td>317, 179, 299, 151, 271</td>
<td>556</td>
<td>c, f, g, h</td>
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<tr>
<td>Quercetin-3-rutinoside</td>
<td>8.02</td>
<td>609</td>
<td>301, 271, 256, 279, 151</td>
<td>354</td>
<td>c, g</td>
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<td>Quercetin-3-hexoside</td>
<td>8.75</td>
<td>463</td>
<td>300, 271, 255, 179, 151</td>
<td>357</td>
<td>b, c, f, g, h</td>
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<td>Quercetin-3-glucuronide</td>
<td>8.93</td>
<td>477</td>
<td>301, 151, 179, 274, 283</td>
<td>354</td>
<td>c, f, g, h</td>
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<tr>
<td>Kaempferol-3-hexoside</td>
<td>10.70</td>
<td>447.5</td>
<td>284, 205, 227, 183, 135, 197</td>
<td>346</td>
<td>c, g</td>
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<td>Kaempferol-3-glucuronide</td>
<td>11.70</td>
<td>447.3</td>
<td>284, 255, 227, 153, 179, 241</td>
<td>346</td>
<td>c, g</td>
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<td>Isohamnetin-3-hexoside</td>
<td>12.60</td>
<td>477</td>
<td>315, 285, 299, 243, 151, 179</td>
<td>354</td>
<td>f</td>
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<td>Isohamnetin-3-glucuronide</td>
<td>13.30</td>
<td>491</td>
<td>315, 300, 271, 255, 179, 151</td>
<td>353</td>
<td>g</td>
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**Phenolic acids**

<table>
<thead>
<tr>
<th>Name</th>
<th>Iq (min)</th>
<th>[M-H] (m/z)</th>
<th>Product Ions (m/z)</th>
<th>λ_{max} (nm)</th>
<th>Detected in</th>
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<tr>
<td>Gallic acid hexoside</td>
<td>6.76</td>
<td>331</td>
<td>271, 211, 169, 151, 125</td>
<td>276</td>
<td>a, c, d, f, g, h</td>
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<tr>
<td>Protocatechuic acid hexoside</td>
<td>7.38</td>
<td>315</td>
<td>153, 123</td>
<td>278</td>
<td>c, d, f, g, h</td>
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<tr>
<td>Ferulic acid hexoside</td>
<td>8.09</td>
<td>355</td>
<td>193, 165</td>
<td>275</td>
<td>c, g</td>
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<tr>
<td>Chlorogenic acid</td>
<td>8.55</td>
<td>353</td>
<td>191, 179, 161, 135</td>
<td>320</td>
<td>a, g</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>10.17</td>
<td>311</td>
<td>179, 149, 135</td>
<td>328, 301(d)²</td>
<td>b, a, c, d, f, g, h</td>
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<tr>
<td>p-Coumaric acid</td>
<td>13.47</td>
<td>295</td>
<td>163, 149, 119</td>
<td>311, 301(d)²</td>
<td>b, c, d, f, g, h</td>
</tr>
</tbody>
</table>

**Flavan-3-ols**

<table>
<thead>
<tr>
<th>Name</th>
<th>Iq (min)</th>
<th>[M-H] (m/z)</th>
<th>Product Ions (m/z)</th>
<th>λ_{max} (nm)</th>
<th>Detected in</th>
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<tbody>
<tr>
<td>Catechin dimer</td>
<td>9.01</td>
<td>577</td>
<td>451, 425, 407, 289</td>
<td>280</td>
<td>a, b, c, e, d, f, g, h</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>11.93</td>
<td>289</td>
<td>245, 203, 179, 161, 125, 137</td>
<td>280</td>
<td>b, c, d, e, f, g, h</td>
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<tr>
<td>(-)-Epicatechin</td>
<td>14.40</td>
<td>289</td>
<td>245, 203, 179, 151, 137, 123, 109</td>
<td>279</td>
<td>b, c, d, e, f, g, h</td>
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</table>

Table 2. Concentration of phenolic compounds in musts and extracts from Pais and Lachryma Christi grapes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Must</th>
<th>Pomace Extract</th>
<th>Leaf Extract</th>
<th>Cane Extract</th>
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<tr>
<td></td>
<td>PA</td>
<td>LC</td>
<td>PA</td>
<td>LC</td>
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<td></td>
<td>PA</td>
<td>LC</td>
<td>PA</td>
<td>LC</td>
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<tr>
<td></td>
<td>PA</td>
<td>LC</td>
<td>PA</td>
<td>LC</td>
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<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
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<tr>
<td>Delphinidin-3-glucoside</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>Petunidin-3-glucoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>n.d.</td>
<td>1419 ± 1 b</td>
<td>97.5 ± 0.8 e</td>
<td>n.d.</td>
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<tr>
<td><strong>Flavonols</strong></td>
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<td></td>
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<tr>
<td>Quercetin-3-rutinoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Quercetin-3-hexoside</td>
<td>n.d.</td>
<td>214 ± 0.4 c</td>
<td>384 ± 0.7 c</td>
<td>n.d.</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kaempferol-3-hexoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kaempferol-3-glucuronide</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Isohamnetin-3-hexoside</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Isohamnetin-3-glucuronide</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Phenolic acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic acid hexoside</td>
<td>1.7 ± 0.0 d</td>
<td>n.d.</td>
<td>21 ± 0.1 c</td>
<td>n.d.</td>
</tr>
<tr>
<td>Protocatechuic acid hexoside</td>
<td>n.d.</td>
<td>2.8 ± 0.0 c</td>
<td>4.6 ± 0.1 b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ferulic acid hexoside</td>
<td>n.d.</td>
<td>4.2 ± 0.0 a</td>
<td>3.9 ± 0.0 b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>1.7 ± 0.0 b</td>
<td>n.d.</td>
<td>11.7 ± 0.9 b</td>
<td>n.d.</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>4.0 ± 1.6 e</td>
<td>n.d.</td>
<td>36.6 ± 1.0 c</td>
<td>n.d.</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>n.d.</td>
<td>8.3 ± 0.0 c</td>
<td>2.6 ± 0.0 e</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flavan-3-ols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechin dimer</td>
<td>7.8 ± 1.0 c</td>
<td>5.9 ± 0.9 c</td>
<td>10.9 ± 2.5 c</td>
<td>n.d.</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>n.d.</td>
<td>54.2 ± 0.2 c</td>
<td>42.3 ± 0.1 f</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

1 Data are expressed as mean ± standard deviation in mg/kg fresh weight (n = 2). Different letters in the same row indicates statistically significant difference (p < 0.05). PA: cultivar Pais, L.C: cultivar Lachryma Christi.
Vine leaves are able to enrich grape musts in flavonols, phenolic acids, and flavan-3-ols (Table 2). However, the availability of flavonols, flavan-3-ols and other flavonoids in grapevine parts depends on grape variety, stage of maturation, and recollection [38]. Concerning the flavonols, quercetin-3-hexoside was the most abundant species followed by quercetin-3-glucuronide, whereas myricetin and kaempferol derivatives were found at lower levels. Moreover, quercetin-3-rutinoside, kaempferol-3-hexoside, kaempferol-3-glucoside, and isorhamnetin-3-glucuronide were exclusively found in leaf extracts, being categorized as leaf-associated components. However, kaempferol-3-glucoside has been detected before in very low concentrations (0.001 mg/g fresh weight) in *V. vinifera* grapes [39]. Furthermore, leaves did not supply additional anthocyanins to grape musts. Additionally, thermomaceration provokes a significant loss of anthocyanins (derivatives of peonidin and malvidin) in LC leaf extracts. The concentration of anthocyanins retained in leaf extracts depends on a combination of thermo-induced effects, including co-pigmentation with flavonols, condensation and polymerization with flavan-3-ols, partitioning between leaves and must, and adsorption to the solid phase [34].

Grapevine canes are a less promising source of polyphenols to enrich grape must than grape pomace or grapevine leaves. Polyphenol extraction from canes provided just a slight increase in the concentration and number of compounds (Table 2). Caftaric, protocatechuic, and coumaric acid were the predominant phenolic acids provided by PA and LC canes, respectively. The flavonols myricetin-3-hexoside, quercetin-3-hexoside, and quercetin-3-glucuronide, which are present at low concentrations, were from cane origin in case of Lachryma Christi. However, these compounds were also detected in pomace and leaves (Table 1). The presence of these compounds was in agreement with previous reports identifying caftaric acid, epicatechin, and quercetin and malvidin derivatives as the main metabolites concerning grape stems [40,41]. Although the presence of stilbenes in grape canes has been reported previously [42–44], these compounds were not detected in this study. The absence of stilbenoids in grapevine extracts can be attributed to the use of polar aqueous extraction conditions and the lack of UV-C irradiation in grapevines, as the biosynthesis of resveratrol in grape leaves is strongly increased in response to UV-C irradiation [45], or the storage at −20 °C of grapevine canes after collection. *E*-resveratrol levels in fresh cut canes that have been kept frozen were very low, whereas post-pruning storage at room temperature induced *E*-resveratrol biosynthesis, giving a significant rise of *E*-resveratrol levels after several months [43].

### 3.2. Physicochemical Characterization

Physicochemical differences were observed between enriched grape juice, i.e., a mixture of pomace, leaf, and cane extracts, and base grape musts due to the extraction conditions and the addition of compounds from leaf, cane, and pomace (Table 3).

**Table 3.** Physicochemical properties and chemical composition of grape musts and enriched juice.

<table>
<thead>
<tr>
<th></th>
<th>PA Must</th>
<th>LC Must</th>
<th>Enriched Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.17 ± 0.01 b</td>
<td>2.88 ± 0.01 c</td>
<td>3.43 ± 0.02 a</td>
</tr>
<tr>
<td>Total acidity (g/kg)</td>
<td>2.46 ± 0.03 c</td>
<td>4.81 ± 0.05 a</td>
<td>2.73 ± 0.00 b</td>
</tr>
<tr>
<td>Soluble solids (Brix)</td>
<td>17.8 ± 0.0 a</td>
<td>15.9 ± 0.0 c</td>
<td>17.5 ± 0.1 b</td>
</tr>
<tr>
<td>Free SO₂ (mg/kg)</td>
<td>9.6 ± 0.0 b</td>
<td>25.6 ± 3.2 a</td>
<td>1.5 ± 0.0 c</td>
</tr>
<tr>
<td>Total SO₂ (mg/kg)</td>
<td>19.2 ± 0.0 b</td>
<td>41.6 ± 2.8 a</td>
<td>1.7 ± 0.3 c</td>
</tr>
<tr>
<td>Color intensity</td>
<td>1.49 ± 0.01 c</td>
<td>2.95 ± 0.03 b</td>
<td>4.64 ± 0.09 a</td>
</tr>
<tr>
<td>Hue—tint</td>
<td>1.37 ± 0.02 a</td>
<td>0.40 ± 0.01 c</td>
<td>0.92 ± 0.02 b</td>
</tr>
<tr>
<td>Total polyphenols (mg/kg)</td>
<td>763 ± 17 c</td>
<td>2015 ± 170 a</td>
<td>1559 ± 59 b</td>
</tr>
<tr>
<td>Total flavonoids (mg/kg)</td>
<td>711 ± 66 b</td>
<td>995 ± 351 ab</td>
<td>1326 ± 32 a</td>
</tr>
<tr>
<td>Monomeric anthocyanins (mg/kg)</td>
<td>0.70 ± 0.00 c</td>
<td>218 ± 8 a</td>
<td>61.0 ± 3.7 b</td>
</tr>
<tr>
<td>ABTS (mmol/kg)</td>
<td>9.59 ± 0.98 b</td>
<td>21.5 ± 4.6 b</td>
<td>77.2 ± 11.1 a</td>
</tr>
</tbody>
</table>

\(^1\) Data are expressed as mean ± standard deviation (n = 3). Different letters in the same row indicates statistically significant difference (p < 0.05). PA: cultivar Pais, LC: cultivar Lachryma Christi.
Unripe grapes were used to prepare musts according to ripeness criteria of soluble solids, acidity, and pH for wine grapes [46]. Increased pH values and a relatively low total acidity found in enriched juice may be due to the loss of volatile compounds during thermomaceration. On the other hand, Lachryma Christi grapes result in an unusual juice that has been characterized by a relatively low sugar content, high acidity, low pH, and high color intensity. Moreover, these grapes are a better source of phenolics, in particular, monomeric anthocyanins and total polyphenols, when compared to Pais grapes (Table 3). Additionally, total antioxidant capacity evaluated by ABTS$^+$ scavenging showed a significant increase for enriched juice when compared to starting materials. These results show the potential of grape pomace, grapevine leaves and canes’ residues as new resources of antioxidants to enrich fruit juices by using green extraction techniques. Relatively high free bisulfite concentrations of base grape musts are important to stabilize them by preventing enzymatic browning and the deterioration of aroma perception. Bisulfite is able to protect grape musts against enzymatic oxidation by polyphenol oxidase via the formation of 2-S-glutathione caftaric acid, which is a relatively stable grape reaction product [47]. This protective role may avoid the formation of O-quinones derived from hydroxycinnamic acids and catechins that are known to react easily with cysteinylated aroma precursors from grape juice and skins affecting aroma stability, as well as the formation of brown oligomers [48]. Since the solid fractions used in juice enrichment contain additional flavonoids, this results in a decline in protective bisulfite contents. Moreover, the relatively high process temperature and surface aeration by intense stirring may facilitate some loss of bisulfite as well.

Analysis of color according to intensity and hue shows differences among samples. Pais had a more yellow hue, while the color of Lachryma Christi and fortified juice samples shifted towards reddish hue. Values of hue-tint and monomeric anthocyanin concentrations showed an inverse linear relationship (Pearson coefficient $r = -0.976$; $p < 0.01$). Anthocyanins and in particular malvidin-3-glucoside were the main pigments in grape juice with a positive correlation between malvidin-3-glucoside concentration and color stability [49]. The relative loss of monomeric anthocyanins after thermomaceration for enriched juice, in combination with an increase of color intensity, indicates co-pigmentation between anthocyanins and flavonoids extracted from skins, seeds, leaves, and canes. Co-pigmentation is an important phenomenon in the case of Teinturier grape cultivars [34]. Acylated anthocyanins are the main pigments of the co-pigmentation complex with cofactors, such as hydroxycinnamic acids (coumaric, caffeic, and ferulic acid), flavonol derivatives (myricetin, quercetin, and kaempferol), or flavone derivatives (vitexin and orientin) [34].

### 3.3. Phenolics as Antioxidant Agents

The antioxidant capacity of enriched juice in this study, as measured by the ability of antioxidants to scavenge ABTS$^+$ radicals, was found ~3.6- and ~8.0-fold higher than for base grape musts (Table 3) and exceeded the values reported for commercial white, red, and purple grape juices at least three-fold [9,50]. Values of antioxidant capacity did not show a linear relationship with monomeric anthocyanin contents (Pearson coefficient $r = -0.080$; $p = 0.837$) and total polyphenol contents ($r = 0.317$; $p = 0.407$), contrary to flavonoid contents ($r = 0.743$; $p = 0.022$). The increase of antioxidant capacity was stronger than the increment of monomeric anthocyanins and total polyphenols found after thermomaceration. This suggests that grape pomace, grapevine leaves and canes contain specific phenolics, whose molecular structures show a stronger antioxidant capacity than those that are contained in base grape musts, or that synergism occurs between them. Both the configuration and the number of hydrogen-donating hydroxyl groups are the main structural features influencing the antioxidant activity of polyphenols. For example, the high activity of catechin dimers among flavan-3-ols was attributed to their hydroxyl functional groups that are potent hydrogen donators [13]. Additionally, the ortho-dihydroxy structure on the B-ring, the 2-3-double bound conjugated with a 4-oxo function in the C-ring, and the free hydroxyl groups in position 3 in the C-ring and position 5 in the A-ring are important structural features for flavonoids [13]. This may explain the relatively high antioxidant activity of quercetin among flavonols, while the glycosylation of hydroxyl substituents
on C3 will drop the antioxidant activity when compared to the aglycon [51]. Conjugated double bonds in combination with a planar molecular structure of quercetin allow for electron delocalization across the molecule, thus stabilizing the corresponding phenoxy radicals [52]. In our study, both catechin dimers and quercetin derivatives increased in grapevine extracts, being the main ingredients of enriched grape juice. In addition, polyphenols are believed to scavenge free radicals by two major mechanisms: by reduction via electron transfer and by hydrogen atom transfer, which may occur in parallel [53]. The CUPRAC assay determines the antioxidant capacity of hydrophilic and lipophilic dietary polyphenols in vitro based on the single electron transfer principle. The ORAC assay evaluates the capacity of antioxidants to inhibit bleaching of a target molecule (probe) induced by peroxyl radicals according to the principle of hydrogen atom transfer. Therefore, it is important to run multiple antioxidant methods, rather than just the ABTS method to get a better estimate of antioxidant potency of phenolic-rich foods on human health.

Enriched grape juice exhibited ORAC-FL values of 19.6 ± 0.0 mmol/kg, being similar to those reported for red grape juices (14.6–25.0 mmol/kg) [53], while ORAC-PGR values (13.7 ± 0.1 mmol/kg) were between those that were reported for white and red wines [30]. When comparing both assays, the stoichiometry of reactions is more important than the reactivity of antioxidants for ORAC-FL, while the absence of induction times in the kinetic profiles of PGR consumption would imply that the ORAC-PGR index is more related to the reactivity of antioxidants than to stoichiometric factors. ORAC-FL is a measure of the amount of reactive polyphenols available, while ORAC-PGR are influenced by the quality of antioxidants present in the sample. The ORAC-PGR/ORAC-FL ratio would reflect the average quality of antioxidants present in a beverage. A high value of this ratio would imply that a high proportion of antioxidants that are contained in the sample are able to protect PGR against bleaching induced by peroxyl radicals. The ORAC-PGR/ORAC-FL ratio of 0.70 for enriched grape juice was similar to that of red, rosé, and white wines [54], but was 5 and 45 times higher than the values of tea and herbal infusions, respectively [55]. Thus, the reactivity of polyphenols that are present in enriched grape juice is at least as efficient as those present in other beverages that are rich in antioxidants.

According to the CUPRAC assay, the antioxidant capacity of enriched grape juice was 3.62 ± 0.20 mmol Trolox equivalents/kg sample. CUPRAC-measured antioxidant capacity was significantly lower than the ORAC values, indicating that enriched grape juice has a more potent radical scavenging capacity by hydrogen atom transfer than reducing capacity via electron transfer. Similar findings were reported after comparing ABTS- and FRAP-measured antioxidant capacities [9].

3.4. Sensorial Evaluation

The results of sensorial tests for LC extracts showed a significantly lower preference score of panelists for leaf extract than for pomace or cane extracts (Table 4).

Strong herbal, tea-like notes and astringency of leaf extracts were considered as negative attributes. These attributes were less notorious in case of PA extracts due to an increased sweetness, lower acidity, and greater aroma complexity that may suppress negative sensory perception. High amounts of flavan-3-ols found in LC leaf extracts can affect taste of this beverage. These compounds, in particular oligomeric tannins, are related to astringency and bitterness [56].

As evidenced by the preference ranking scores (Table 4), LC juice supplemented with pomace, leaves and canes at a ratio of 40:20:40 (v/v/v) was more attractive than other samples giving a complex aroma and a well-balanced sweet acid taste, combined with a slight astringency. In addition, half of País and half of Lachryma Christi juice enriched with pomace, leaves and canes at a ratio of 40:20:40 (v/v/v) should be mixed to yield a well-balanced taste of sweetness and acidity, together with slight notes of a vegetal-woody odor and a reddish hue with increased color intensity.
Table 4. Preference ranking score for blends of pomace, leaves and canes extracts \(^1\).

<table>
<thead>
<tr>
<th>First Level Target</th>
<th>Second Level Target</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pais</td>
<td>Pomace</td>
<td>7.5 ± 1.2 (^a)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>4.0 ± 1.0 (^a)</td>
</tr>
<tr>
<td></td>
<td>Cane</td>
<td>3.5 ± 0.9 (^a)</td>
</tr>
<tr>
<td>Lachryma Christi</td>
<td>Pomace</td>
<td>6.5 ± 1.1 (^a)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>1.0 ± 0.6 (^b)</td>
</tr>
<tr>
<td></td>
<td>Cane</td>
<td>7.5 ± 1.3 (^a)</td>
</tr>
<tr>
<td>Pais</td>
<td>Pomace/leaf/cane ratio of 25/25/50</td>
<td>5.0 ± 0.5 (^a)</td>
</tr>
<tr>
<td></td>
<td>Pomace/leaf/cane ratio of 40/20/40</td>
<td>3.0 ± 0.5 (^a)</td>
</tr>
<tr>
<td></td>
<td>Pomace/leaf/cane ratio of 50/17/33</td>
<td>2.0 ± 0.4 (^a)</td>
</tr>
<tr>
<td>Lachryma Christi</td>
<td>Pomace/leaf/cane ratio of 25/25/50</td>
<td>1.0 ± 0.3 (^b)</td>
</tr>
<tr>
<td></td>
<td>Pomace/leaf/cane ratio of 40/20/40</td>
<td>7.0 ± 0.5 (^a)</td>
</tr>
<tr>
<td></td>
<td>Pomace/leaf/cane ratio of 50/17/33</td>
<td>2.0 ± 0.4 (^b)</td>
</tr>
<tr>
<td>Pomace/leaf/cane ratio of 40/20/40</td>
<td>Pais/Lachryma Christi ratio of 90/10</td>
<td>0.8 ± 0.2 (^b)</td>
</tr>
<tr>
<td></td>
<td>Pais/Lachryma Christi ratio of 50/50</td>
<td>5.4 ± 0.5 (^a)</td>
</tr>
<tr>
<td></td>
<td>Pais/Lachryma Christi ratio of 70/30</td>
<td>3.9 ± 0.5 (^ab)</td>
</tr>
</tbody>
</table>

\(^1\) Different letters in the same block indicates statistically significant difference (\(p < 0.05\)).

4. Conclusions

The polyphenol-rich grape juice, made by thermomaceration using grape pomace, grapevine leaves and canes, shows a promissory Trolox equivalent antioxidant capacity of 77.2 mmol/kg juice, according to the ABTS assay that may offer health benefits. Catechin dimers and quercetin derivatives with high antioxidant activity are main ingredients of enriched grape juice. Both grape pomace and grapevine leaves are of primary importance as additional polyphenol sources in the preparation of enriched grape juice. Pomace and leaf extracts of both Pais and Lachryma Christi cultivars evidenced an elevated concentration of phenolic compounds, which is largely attributed to their anthocyanin, flavonol, and flavan-3-ol content.

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Author Contributions: Tabita Aguilar conceived and designed the experiments; Cristina Loyola, Luis Bustamante and Carola Vergara performed the experiments; Tabita Aguilar, Dietrich von Baer, Ignacio Serra and Johannes de Bruijn analyzed the data; Claudia Mardones contributed reagents/materials/analysis tools; Johannes de Bruijn wrote the paper.

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

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