Anthocyanin-rich extracts from purple and red potatoes as natural colourants:

bioactive properties, application in a soft drink formulation and sensory analysis

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Abstract

Aqueous extracts from seven coloured potato varieties (three red-fleshed, three-purple fleshed, and one marble-fleshed genotype) were studied for their anthocyanin content, *in vitro* biological activities, colouring properties and their potential application in the food industry. Acylated glycosides or pelargonidin and petunidin aglycones were identified as the main anthocyanin forms in the red and purple varieties, respectively. The total anthocyanin content among varieties ranged from 478.3 to 886.2 mg/100 g extract. All the extracts presented *in vitro* antioxidant, antibacterial and antifungal activities, whereas no toxic effects were detected. Finally, two selected extracts were tested as colourants in a soft drink formulation and presented suitable sensory profiles as well as high colour stability during a 30-day shelf-life when compared with the commercial colourant E163. Therefore, the tested extracts could be used as natural food colourants.

1. Introduction

There is a growing interest worldwide in the development of food colourants from natural sources as a consequence of perceived consumer preferences and the concerns about the use of synthetic compounds in food products (Albuquerque, Pinela, Barros, Oliveira, & Ferreira, 2020; Petropoulos et al., 2019). In response to that, the food and beverage industries have been seeking natural colouring agents that could substitute synthetic dyes and colouring additives (Petropoulos et al., 2019; Sampaio et al., 2020b; Schieber, Stintzing, & Carle, 2002).

Coloured root vegetable products can be alternative sources of colouring and bioactive compounds (Hossain, Rawson, Aguiló-Aguayo, Brunton, & Rai, 2015; Nemś & Pęksa, 2018). Among root vegetables, potato (*Solanum tuberosum* L.) is the most consumed worldwide, being a staple for 1.3 billion people and with an increasing popularity in the developing world (Stokstad, 2019). The nutritious tuber also presents the highest genetic diversity among all cultivated species, with approximately 5000 registered varieties and a broad phenological variation in terms of flesh and skin colour (Petropoulos et al., 2019). Red and purple-fleshed potatoes are rich in phenolic compounds, particularly in anthocyanins, presenting about three times higher amounts of total phenolic compounds content than the widely consumed white- and yellow-fleshed tubers, as well as two to three times higher antioxidant activity (Petropoulos et al., 2019). Anthocyanins are suitable to be used as natural colouring compounds due to their bright attractive red and purple colours and water solubility that allows for their easy incorporation into aqueous food systems (Rodriguez-Saona, Giusti, & Wrolstad, 2008; Rodríguez-Saona, Glusti, & Wrolstad, 1999). Nevertheless, they can pose problems of colour instability when they are out of their natural environment, as affected by factors such as pH, temperature, light, oxygen or interactions with other coexisting components, among others (Santos-Buelga

& González-Paramás, 2019). The anthocyanin molecules found in coloured potatoes are usually acylated with *p*-coumaric or ferulic acids. The acylation of the anthocyanin molecules improves pigment stability during processing and storage (Rodriguez-Saona, Giusti, & Wrolstad, 1998). This effect is chemically explained by the stacking of the acyl groups with the pyrylium ring of the flavylium cation, which reduces the susceptibility of the nucleophile attack of water on the hydrophilic anthocyanin molecules and the formation of a colourless pseudobase and light yellow chalcone forms (Sasaki, Nishizaki, Ozeki, & Miyahara, 2014; Santos-Buelga & González-Paramás, 2019).

Natural anthocyanins have powerful colouring capacities and at acidic pH values only small concentrations of these compounds are required to obtain the desired red, pink and purple colours in food products (Mateus & Freitas, 2009). The list of applications of anthocyanins as colouring agents is long, comprising products such as fruit preservatives (Bursać Kovačević et al., 2015), sugar confectionary (Mateus & Freitas, 2009), dairy products (Montibeller, de Lima Monteiro, Tupuna-Yerovi, Rios, & Manfroi, 2018; Pires et al., 2018), dry mixes (acid dessert mixes and drink powders) (Mateus & Freitas, 2009), frozen products (ice creams) (Mateus & Freitas, 2009), bakery products (Albuquerque et al., 2020; Da Silva et al., 2019a, 2019b) and beverages (Monteiro et al., 2017; Montibeller et al., 2018). Among all possible applications, soft drinks are suitable candidates due to their acidic pH (below 3.5) and the high solubility of anthocyanins in water (Mateus & Freitas, 2009; Montibeller et al., 2018). Nevertheless, these molecules can be affected by heat processing, hence the importance of testing the colour stability after pasteurisation, as soft drink formulations may be heat-processed to prolong the product's safety during shelf-life.

In this work we present an in-depth characterisation of the anthocyanin profile and the bioactivities (*in vitro* antioxidant, antibacterial and antifungal activities) of aqueous

extracts from six varieties of red and purple-fleshed potatoes, as well as a marble-fleshed cultivar being used for comparison purposes. Furthermore, a cytotoxicity assay to test the safety of the extracts was carried out, and then two varieties (one red and one purple) that presented the most promising results were selected for an application study by incorporating their extracts in a pasteurised soft drink model. Finally, the sensory profile of the products was assessed by quantitative descriptive analysis (QDA) and the colour stability of the produced soft drinks was monitored over a 30-day shelf-life period, comparing the performance of the extracts with the commercial colourant E163.

a sensory evaluation (quantitative descriptive analysis) and a colour stability assessment of the produced soft drinks over a 30-day shelf-life period is presented, comparing the performance of the extracts with the commercial colourant E163.

2. Materials and methods

2.1 Plant material

Twenty potato seeds (tubers) from the tested potato varieties (*Solanum tuberosum* L.), namely three red-fleshed genotypes (cv. *Rosemary, Red Emmalie,* and *Red Cardinal*), three purple-fleshed genotypes (cv. *Purple, Violetta*, and *Kefermarkter Blaue*), and one marble-fleshed genotype (cv. *Shetland Black*) were purchased from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK). The obtained tubers were cultivated in the experimental farm of the University of Thessaly in Velestino, Greece, according to commercial cultivation protocols during the spring-summer growing period of 2018. Seed tubers were planted manually on 3/4/2018 with distances of 75 cm between hills and 30 cm within each hill and approximately 10 cm depth (Petropoulos et al., 2020). Fertilizers were applied with base-dressing (400 kg/ha of 15-15-15 (N-P-K fertilizer) and two side-dressings (1st: 50 kg/ha of ammonium nitrate; 2nd 50 kg/ha of potassium nitrate). Irrigation

was applied via a sprinkler irrigation system, while weeds and pests were controlled according to the best practice guides for the crop. Tubers were harvested manually on 27/7/2018. The fresh harvested tubers (**Figure 1**) were transported to the Polytechnic Institute of Bragança, Portugal, where the samples were washed with cold water upon arrival to eliminate extraneous matter and refrigerated at 4 ºC until analysis.

2.2 Extracts preparation

Washed potato tubers were manually peeled and immediately immersed in an aqueous citric acid solution (0.5 mol/L) to prevent browning (1 g fresh tuber/2 mL citric acid solution). After the immersion in the citric acid solution, the whole peeled tubers were gently blended (commercial juice blender, Breville Blend Active model VBL134) and filtered twice (Whatman No. 4 paper). The obtained acidified aqueous extracts (pH ~3) were freeze-dried (-49 ºC, 0.08 bar, during 48 h, FreeZone 4.5 model 7750031, Labconco, Kansas, USA). All seven resulting extracts presented a yield of approximately 5% (mass of freeze-dried extract/mass of whole fresh potato). All steps in the extraction process were carried out in food grade facilities.

2.3 Determination of anthocyanin content and bioactivities

2.3.1 Chromatographic analysis of anthocyanins

The seven freeze-dried extracts were dissolved in water to a concentration of 50 mg/mL and filtered through 0.22 μm disposable syringe filters into amber vials for high performance liquid chromatography (HPLC) analysis. The analysis was performed using a Dionex Ultimate 3000 HPLC (Thermo Scientific) system equipped with a quaternary pump, an automatic injector (at 5 °C), a degasser, and an automated thermostat column compartment. The compounds detection was carried out with a diode-array detector

(DAD), using 520 nm as preferred wavelength, and electrospray ionisation (ESI) coupled to a mass spectrometry (MS) detector (HPLC-DAD-ESI/MS), operating under the conditions described by Albuquerque, Pinela, Barros, Oliveira, & Ferreira, (2020). Data were collected and analysed using the Xcalibur® program (Thermo Scientific). The identification was performed from the chromatographic retention characteristics and UV-Vis and mass spectra, and comparison with data described in the literature. The quantification was performed using a 7-level calibration curve of peonidin-3-O-glucoside ($y = 151438x - 3E^{+06}$, $R^2 = 0.9977$, LOD = 0.20 μg/mL and LOQ = 0.71 μg/mL; Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as mg per 100 g of extract.

2.3.2 Antioxidant activity

The antioxidant activity of the seven extracts was assessed in terms of their potential to inhibit the production of thiobarbituric acid reactive substances (TBARS) in brain homogenates, a cell-based *in vitro* method previously described by Sampaio et al. (2020a). The formation of the complex malondialdehyde-thiobarbituric acid was measured at 532 nm and the results expressed as IC_{50} values (mg/mL of extract).

2.3.3 Cytotoxicity evaluation

The seven potato extracts were dissolved in water (4 mg/mL) and submitted to further dilutions. A cell culture (PLP2) was prepared using a freshly harvested porcine liver according to the method described by Corrêa *et al.* (2015). The sulforhodamine B assay was performed to evaluate any possible cytotoxicity of the extracts. Ellipticine (Sigma-Aldrich, St. Louis, MO, USA) was employed as a positive control and the results were expressed in GI_{50} values.

2.3.4 Antibacterial and antifungal activities

With the aim to assess the potential use of the potato extracts as natural food preservatives, their antibacterial and antifungal activities were evaluated according to the procedure described by Corrêa et al. (2015). Three Gram-positive bacteria (*Bacillus cereus* (clinical isolate), *Staphylococcus aureus* (ATCC 11632) and *Listeria monocytogenes* (NCTC 7973)) and three Gram-negative bacteria (*Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella* Typhimurium (ATCC 13311)) were used to determine the potential antibacterial activity of the extracts. For the antifungal assays, six micromycetes were tested, namely *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061) and *Penicillium aurantiogriseum* (food isolate). Two commercial food preservatives were used as positive controls: potassium metabisulphite (E224) and sodium benzoate (E211). Results were given as minimal inhibitory, bactericidal and antifungal concentrations (MIC, MBC, MFC, respectively).

2.4 Validation of the anthocyanin-rich extracts as natural colourants

2.4.1 Soft drink formulation

Following the screening of the seven potato varieties based on their anthocyanin content and bioactive properties, two extracts were selected to be applied as colouring agents in a soft drink formulation: cv. *Rosemary* (red-flesh potato variety) and cv. *Purple* (purple-fleshed potato variety). A typical raspberry flavoured soft drink formulation was prepared, composed of sparkling water, caster sugar, citric acid, sucralose and natural flavourings (raspberry and peach), in decreasing order of concentration. Three soft drink formulations were prepared with (1) the red anthocyanin-rich potato extract (*Rosemary*),

(2) the purple anthocyanin-rich potato extract (*Purple*) and (3) the commercial colorant E163 (standardised in 1% anthocyanins content). The latter formulation (3) was used as control. The colourants were added to the respective formulations in order to obtain a similar concentration of anthocyanins (~8 mg/L solution), which visually matched the colour intensity of a commercial soft drink product used as reference. The commercial colorant E163 used as control in the soft drink formulations was purchased from Naturex (Derbyshire, UK), consisting of an anthocyanin glucosides' extract derived from grapes and standardised in 1% of anthocyanins content by the supplier, preserved with potassium sorbate. All soft drink samples were prepared according to good hygiene and manufacturing practices in food grade facilities.

2.4.2 Pasteurisation

The three soft drink formulations (*Rosemary*, *Purple* and *Control*) were poured into glass containers (250 mL), which were sealed and placed in a water bath at 70 °C for 20 minutes, following the *in-pack* pasteurisation method described by Ashurst (2011).

2.4.5 Colour stability and determination of pH

Following pasteurisation, the three soft drink formulations (*Rosemary*, *Purple* and *Control*) were stored at 4 °C and their colour stability was assessed at 0, 7 and 30 days of storage. A PCE colourimeter (model CSM-3; PCE Instruments, Germany) was used to measure the colour by placing 80 mL of each formulation in a quartz glass box designed for measurements carried out on liquid samples. Using a silicon photoelectric diode sensor and a measuring aperture of 8 mm, the following CIELab colour space readings were measured through the computerized system: L^*: lightness from black (0) to white

(100); a*: green ($-$) to red ($+$); b*: blue ($-$) to yellow ($+$); C*: chroma, relative saturation; and h°: hue angle in the CIELab colour wheel.

The pH of the formulated soft drinks was measured using a calibrated digital pH meter (portable food and dairy pH meter HI 99161, Hanna Instruments, Woonsocket, RI, USA). All pH measurements were carried out in triplicate. All three soft drink formulations presented a final pH of approximately 3.

2.4.4 Sensory analysis

A sensory analysis was carried out immediately after the soft drinks' production and pasteurisation (Day 0). Sessions took place in the sensory suite facility of the Scottish Centre for Food Development and Innovation (SCFDI) at Queen Margaret University (Edinburgh, UK). The test panel participants were informed about the general aim of the study and the required procedures for handling personal data. All participants gave written informed consent prior to participation. Twelve experienced trained panellists were selected and subsequently received specific additional training on the sensory attributes relevant to the soft drink formulation developed in this project using a range of commercial products. Sensory tests were conducted under white light in each booth.

The soft drinks formulated with the two extracts obtained in the present study were compared with those of a control formulation containing the commercial colorant E163. Samples were presented to the panellists in coded clear plastic cups containing 20 mL of each formulation. Panellists were asked to cleanse their palate before the first sample and between samples using water and crackers. After training the following list of sensory attributes was generated and confirmed by the panel: colour intensity, cloudiness, sweetness, fruitiness, bitterness and sourness. A quantitative descriptive analysis (QDA) test was designed using the Compusense Cloud software (Compusense, Guelph, ON,

Canada). The intensity of perception was scored for each attribute on a scale ranging from 1 (very low) to 9 (very high). Following the QDA questionnaire the panellists were asked to comment on the overall acceptability of each product based on their hedonic experience and indicate their preference among the three products.

2.5 Statistical analysis

Three samples were used for each assay, and all assays were carried out in triplicate. The results were analysed using the one-way analysis of variance (ANOVA) followed by Tukey's HSD Test (p = 0.05). All data are expressed as mean values and standard deviation (SD). When less than three results were present in each individual analysis, t-Student test was used to determine the significant difference ($p = 0.05$). The sensory results were analysed using a two-way analysis of variance (ANOVA) followed by a Tukey's post-hoc Honest Significant Difference (HSD) test using the Compusense Cloud software ($p = 0.05$).

3. Results and discussion

3.1 Aqueous extracts

3.1.1 Composition of anthocyanins

The anthocyanins profile, chromatographic characteristics, tentative identifications, and quantification of the seven aqueous extracts are shown in **Table 1**. All tentatively identified compounds were previously described in the literature for red and purple-fleshed potatoes (Alcalde-Eon et al., 2004; Kita, Bąkowska-Barczak, Hamouz, Kułakowska, & Lisińska, 2013; Oertel et al., 2017).

Seven peaks were detected in the red-fleshed varieties, six of which were identified as pelargonidin derivatives, based on the observation of their characteristic fragments in

MS^2 spectra. **Peak 1r** ([M]$^+$ at *m/z* 741) was identified as pelargonidin-3-rutinoside-5-glucoside based on the HPLC-DAD-MS results and previous literature reports (Kita et al., 2013; Oertel et al., 2017), and was the major anthocyanin detected in the *Red Cardinal* extract. **Peak 2r** ([M]$^+$ at *m/z* 579) also showed an MS^2 fragment at *m/z* 271, corresponding to pelargonidin, bearing the loss of a hexose moiety (−162 u) and being assigned as pelargonidin-3-rutinoside. The remaining peaks were identified as acylated anthocyanins owing to the presence of a shoulder in the UV spectra of the compounds around 310-330 nm. **Peaks 3r**, **4r**, **5r**, **6r**, and **7r** were identified as acylated pelargonidin glycosides. Pelargonidin-3-*p*-coumaroylrutinoside-5-glucoside (**Peak 5r**) was the main compound detected in the *Red Emmalie* and *Rosemary* extracts. **Peak 3r**, with the same molecular ion, might correspond to an isomer of peak 5r having the glucoside residue located at position 7, which is expected to elute earlier than the corresponding 5-glucoside, as reported by Alcalde-Eon et al. (2004) for similar petunidin isomers in *Solanum stenotomum* tubers. In agreement with our findings, acylated pelargonidin glycosides have been previously identified by other authors as the main pigments in red potato tubers (Kita et al., 2013; Lewis et al., 1998; Oertel et al., 2017; Rodríguez-Saona et al., 1999).

All three red-fleshed varieties showed similar total anthocyanin content, and the *Red Cardinal* variety presented the highest value (886.2 ± 1.9 mg/100 g extract). Considering the extraction yield of 5% (mass of freeze-dried extract/mass of whole fresh potato), our results are in agreement with those found by Kita et al. (2013) for red-fleshed potato tubers. Moreover, Hamouz et al. (2011) also reported significant differences in total anthocyanin content among red- and purple-fleshed potato cultivars.

Regarding the purple-fleshed varieties, eight peaks were detected, seven of which were tentatively identified as petunidin derivatives (**Peaks 1p** – **6p** and **8p**) and one compound

as an acylated peonidin glycoside (**Peak 7p**). The molecular ions and fragmentation patterns of peaks **4p** to **6p** were coherent with derivatives of petunidin-3-rutinoside-5-glucoside acylated with caffeic, *p*-coumaric and ferulic acids, respectively, and peak **8p** with petunidin-3-*p*-coumaroylrutinoside, all of them previously identified in purple tubers of *Solanum tuberosum* (Kita et al., 2013) and *S. stenotomum* (Alcalde-Eon et al., 2004). Similarly, the characteristics of peak **7p** coincide with those of peonidin-3-feruloylrutinoside-5-glucoside described in purple tubers of *S. stenotomum* (Alcalde-Eon et al., 2004). Peak **3p** possessed the same molecular ion $[M]^+$ at *m/z* 933 as peak **5p**, but different fragmentation pattern. Two compounds with this molecular ion were also reported by Alcalde-Eon et al. (2004) in purple tubers of *S. stenotomum* that were respectively identified as petunidin-3-*p*-coumaroylrutinoside-7-glucoside and petunidin-3-*p*-coumaroylrutinoside-5-glucoside, although in that case both compounds showed similar MS^2 fragmentation. In the present study, peak **3p** produced a main fragment at *m/z* 625, from the loss of 308 mu that could correspond either to a coumaroylglucoside or a rutinosyl residue, and a second fragment at *m/z* 479 (-146 mu) from the further loss of either a coumaroyl or a rhamnosyl residue. This fragmentation rather seems to suggest an identity where the coumaroyl would be located on a glucose moiety instead on the rhamnose moiety, e.g., petunidin-rutinoside-*p*-coumaroylglucoside or petunidin-*p*-coumaroylglucosylrhamnoside-glucoside. Since no definite identity could be assigned this peak was just assigned as a positional isomer of peak **5p**. Finally, peaks **1p** and **2p** showed earlier retention times and their absorption spectra lacked a shoulder at 310-330 nm indicating that they were not acylated. Peak **1p** was assigned as petunidin-3-rutinoside-5-glucoside, previously identified by Kita et al. (2013) and Lewis et al. (1998) in purple tubers of *Solanum tuberosum*. The MS^2 fragmentation of peak **2p** produced ions at *m/z* 479 (-292 mu; loss of two rhamnosyl residues) and 317 (-162 mu; loss of a glucosyl

residue), so that the compound could be assigned as petunidin-rutinoside-rhamnoside, or alternatively to petunidin-dirhamnoside-glucoside. Petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**Peak 5p**) was the main anthocyanin found in the extracts obtained from *Violetta*, *Kefermarkter Blaue* and *Shetland Black* tubers. The same compound was reported by Lewis et al. (1998) and Kita et al. (2013) and as the main anthocyanin in different varieties of purple potatoes, and by Alcalde-Eon et al. (2004) in purple tubers of *S. stenotomum*. In our study, among the four tested purple-fleshed genotypes, *Kefermarkter Blaue* showed the highest total anthocyanin content (533.4 ± 2.8 mg/100 g extract).

The variability in the anthocyanin content among the studied extracts could be mainly attributed to inherited differences of the potato genotypes, as all samples were subjected to the same environmental factors (growing conditions, planting location and climate), cultivation practices, processing and storage conditions. Oertel et al. (2017) reported a great diversity in polyphenol and anthocyanin profiles of red and purple-fleshed potatoes and suggested differences in the enzymatic reactions leading to the hydroxylation and methylation of B-ring of the precursor dihydroflavonols, resulting in the biosynthesis of diverse and species specific anthocyanin backbones. In the same context, Chaves-Silva et al. (2018) attributed the differences in the genetic regulation of biosynthetic pathways through the involvement of different structural and regulatory genes, while Lachman et al. (2012) highlighted the effect of genotype on the anthocyanin content of colour-fleshed potatoes.

3.1.2 Bioactive properties

The *in vitro* antioxidant, antibacterial and antifungal properties of the red and purple aqueous extracts were also evaluated, and the results are presented in **Table 2**.

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Additionally, a hepatotoxicity assay was carried out to assess the safety of the extracts for incorporation into food formulations.

Several studies have demonstrated the antioxidant properties of red and purple potatoes, which have been linked to their high content of anthocyanins (Jayawardana et al., 2012; Kita et al., 2013; Nemś & Pęksa, 2015, 2018). Previous studies have reported positive results for *in vitro* antioxidant assays on coloured potatoes, namely the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the ferric reducing/antioxidant power (FRAP) assays. Herein, a cell-based *in vitro* assay (inhibition of production of TBAR substances) was applied to assess the antioxidant properties of the tested colour-fleshed potato tubers.

As presented in **Table 2**, all seven studied aqueous potato extracts were effective in diminishing the production of TBAR substances, which result from lipid peroxidation-induced breakdown. Among the studied varieties, the purple-fleshed cv. *Violetta* presented the highest antioxidant capacity, as it required the lowest concentration of extract to inhibit the lipid peroxidation process by 50% (IC_{50} value = 380.1 µg/mL) compared to control (Trolox, IC_{50} value = 139±5 µg/mL). For the red-fleshed varieties, cv. *Rosemary* presented the best result (IC_{50} value = 416.9 µg/mL).

In agreement with our findings, Kita et al. (2013) previously reported positive results for antioxidant activity assays carried out on fresh tubers of red and purple-fleshed potatoes, where for instance the purple variety *Salad Blue* showed significant antioxidant activity (ABTS: 1.56 ± 0.2 mM/100 g dw; DPPH: 0.65 ± 0.3 mM/100 g dw; FRAP: 3.17 ± 0.3 mM/100 g dw). Similarly, Nemś et al. (2015) reported a positive antioxidant activity for snacks enriched with coloured potato flour obtained from the same *Salad Blue* variety, employing *in vitro* assays (ABTS: 1.16 ± 0.07 µmol Trolox/g dw; DPPH: 0.60 ± 0.06 µmol Trolox/g dw; FRAP: 0.80 ± 0.07 µmol Trolox/g dw). Nemś and Pęksa (2018), who

also incorporated dried coloured-fleshed potatoes into snacks, reported a beneficial effect on the inhibition of oxidative changes in the lipid profile compared to a control formulation over a 3-months storage period. Moreover, in a study conducted by Jayawardana et al. (2012), the addition of 2% coloured potato flakes into pork sausages suppressed lipid oxidation by 80% compared to the control. Thereby, the antioxidant properties of coloured potatoes could also have a beneficial effect in processed food products by extending their shelf-life.

In order to assess the safety of the studied extracts an *in vitro* cytotoxicity assay was performed. Porcine liver was used as a model, which is justified by its similarity with the human liver in terms of cellular and physiological functioning (Corrêa et al., 2018). The results revealed that all seven aqueous extracts did not present toxicity against the porcine liver primary culture PLP2, as their GI_{50} values were higher than the highest tested concentration for all varieties (400 μg/mL) **(Table 2).** The verified absence of cytotoxicity indicates the safety of the tested extracts for their utilisation as natural food colourants.

The results for antibacterial and antifungal activities are presented in **Table 3**. All seven extracts presented relevant antibacterial and antifungal activities against all the tested bacteria and fungi strains. Cultivar *Red Cardinal* performed particularly well against bacteria, as the required MIC value of the extract against *Staphylococcus aureus* (2 mg/mL) was half the MIC value required by the commercial preservative E211, sodium benzoate (4 mg/mL). The same variety also performed better than the preservative E224 (potassium metabisulfite) against *Bacillus cereus*, as it required half of the MIC and MBC values for this bacterium strain.

All seven aqueous extracts performed equally or better than the preservatives E211 and E224 for all the tested fungi strains. The best results against fungi were achieved by the extract from the *Kefermarkter Blaue* variety, as its antifungal capacity exceeded both

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preservatives for all the tested strains. The obtained results of the antimicrobial properties indicate that all the studied extracts could be useful in retarding and/or suppressing the growth of food borne microbes, which make them suitable candidates for application in food products as natural preservatives.

Previous studies by other authors also reported positive antimicrobial activities from coloured potato extracts. For instance, Ombra et al. (2015) studied the purple-fleshed variety *Vitelotte Noire*, and found its anthocyanin-rich extract was effective against the bacteria strains *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus.* Interestingly, those authors also found that the natural bioactive compounds from purple potatoes retained their biological activity (antimicrobial, antioxidant and anti-proliferative) during their passage through the gastrointestinal tract, suggesting that the long-term consumption of these nutrient components could be beneficial in terms of disease prevention. Similar results were reported by Bontempo et al. (2013), who tested the purple-fleshed variety *Vitelotte* and observed significant activities against *Staphylococcus aureus* and *Rhizoctonia solani*.

3.2 Application of the extracts in soft drink formulations

Following the screening of the seven potato varieties on their anthocyanin profiles and bioactivities, two aqueous extracts were selected to be incorporated as natural colourants into soft drink formulations, namely one from the red-fleshed variety *Rosemary* and one from the purple-fleshed variety *Purple*. Then, the two soft drink formulations were assessed for their sensory attributes and stability after thermal processing (pasteurisation), in comparison with the *Control* colourant E163.

3.2.1 Colour stability assessment

The results for the colour parameters L^* (lightness), a^* (green-redness), b^* (blue-yellowness), C^* (chroma/saturation) and $h°$ (hue) measured over time (after pasteurisation) are presented in **Figure 2**.

At *Day 0* the control soft drink showed a slight distinct colouration when compared to *Rosemary* and *Purple*, presenting lower a^* and higher b^* values, which means a more intense presence of blue tones and less intensity of red. Despite all three formulations having the same concentration of anthocyanins, this difference in colour tone and intensity may be due to the distinct anthocyanin glycosides present in the two tested colouring agents (**Table 1**) and in the control E163 (grape anthocyanins). It should be noted that the colour profile of the three samples presented a great stability between *Day 0* and *Day 7* for all the analysed parameters. At *Day 30* a significant decrease in a^* values was reported for all the tested formulations, which could be related to a reduction in the intensity of the red colour. A strong positive correlation between the total anthocyanins content and the chroma parameters in purple-fleshed sweet potatoes was also observed Loypimai, Moongngarm, & Chottanom (2016). According to Ashurst (2011), in a comprehensive report on the stability and shelf life of fruit juices and soft drinks, product discoloration is a process commonly observed in almost any soft drink or fruit juice. This can be explained by a decrease in the concentration of the original anthocyanins as a result of their degradation and/or transformation in newly-formed derived pigments (Santos-Buelga & González-Paramás, 2019). The degradation of anthocyanins over time was reported by Jie et al. (2013) and Loypimai et al. (2016) in purple-fleshed sweet potato and black rice extracts, respectively. Similarly, anthocyanin decay and a discoloration process was observed by Rodriguez-Saona et al. (1998) when monitoring the anthocyanin stability of frozen red-fleshed potato tubers over a three month-period storage. Those

authors attributed the observed anthocyanin content decay to possible enzymatic reactions.

According to the results herein obtained, the performances of the aqueous potato extracts over shelf-life could be considered satisfactory, as they were similar to the control commercial colourant (a grape extract containing potassium sorbate as preservative). This performance might be explained by the presence of acylated anthocyanins in potatoes. The acylation confers increased biochemical stability to anthocyanin storage forms, preventing their indiscriminate degradation by glycosidases, most of which are unable to act on acylated glycosides (Mateus & Freitas, 2009). Therefore, our results indicate the suitability of the red and purple potato aqueous extracts to be used as alternative natural colouring agents in beverages.

3.2.2 Sensory analysis

The scores obtained in the sensory analysis are shown in **Figure 3**. The *Rosemary*, *Purple* and *Control* formulations were assessed by the 12 trained panellists for the following attributes: intensity of colour, cloudiness, sweetness, fruitiness, bitterness and sourness. Regarding visual attributes, no statistical difference was reported between the *Control* formulation and *Rosemary* for intensity of colour and cloudiness. *Purple*, however, presented a significantly cloudier solution and a significantly lighter colour than both *Rosemary* and *Control* ($p < 0.05$). *Rosemary* and the *Control* formulations were described by the panellists as presenting a light transparent pink colour while *Purple* was described as presenting a slightly opaque light pink colour.

No statistical difference was reported between the three formulations for the flavour attributes sweetness, fruitiness and bitterness. With regards to sourness, *Control* was rated significantly higher than *Rosemary* ($p < 0.05$). Nevertheless, no significant difference was

found between *Purple* and *Rosemary* and between *Purple* and the *Control* for this attribute. Additionally, no off-odours or off-tastes were detected in any of the tested formulations.

At the end of the sensory session the 12 panellists were asked to choose their preferred formulation between the three ones tested. The soft drinks formulated with *Rosemary* and *Purple* extracts performed well, with 42% of the panellists choosing *Purple* and 25% choosing *Rosemary* as their preferred one, while 33% preferred the *Control* soft drink. Interestingly, some panellists highlighted in their comments the opaqueness described in *Purple* as a positive attribute, relating it to a "natural rather than synthetic appearance". The presented results regarding the sensorial analysis and the stability over time of the red and purple-fleshed potato formulations indicate promising potential for their future application in the food industry as substitutes of synthetic colouring agents.

4. Conclusion

The seven aqueous potato extracts tested presented high anthocyanin content and high antioxidant, antibacterial and antifungal properties. Acylated pelargonidin glycosides were the main compounds found in the red varieties and acylated petunidin glycosides in the purple ones. Additionally, no cytotoxic effect was detected in the extracts up to the maximum tested concentration (400 μg/mL), indicating their safety to be incorporated in food formulations. The two extracts selected to be applied in a soft drink formulation showed suitable profiles in the sensory and shelf-life assessments when compared with the control commercial colourant E163. The aqueous extracts herein obtained by a simple one-step and cost-effective extraction method could be used as alternative natural food colourants, substituting synthetic colouring agents. Nevertheless, future studies in pre- and post-harvest level could support the selection of colour-fleshed potatoes with high

anthocyanin content. Furthermore, the assessment of different agronomic practices may increase the concentration of anthocyanins and the added value of this important vegetable crop.

Conflict of interests

The authors declare no conflict of interests.

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Table 1. Chromatographic retention, absorption and mass spectral characteristics, tentative identification, and quantification (mg/100 g extract dw) of anthocyanins found in aqueous extracts from red and purple, potato varieties (mean ± SD).

Red flesh

Peak	Rt (min)	λ_max (nm)	[M]+ (m/z)	MS² (m/z)	Tentative identification	Rosemary	Red Emmalie	Red Cardinal
1r	8.27	499	741	579(100),433(15),271(45)	Pelargonidin-3-rutinoside-5-glucoside	130 ± 1[c]	171 ± 1[b]	264 ± 1[a]
2r	20.0	501	579	433(100),271(46	Pelargonidin-3-rutinoside	109 ± 1[c]	127.9 ± 0.4[b]	206.1 ± 0.1[a]
3r	28.08	500	887	725(100),433(5),271(16)	Pelargonidin-3-p-coumaroylrutinoside-glucoside isomer	106.983 ± 0.003[c]	116 ± 1[b]	214 ± 1[a]
4r	29.86	505	903	741(100),433(16),271(27)	Pelargonidin-3-caffeoylrutinoside-5-glucoside	109.7 ± 0.1	nd	nd
5r	35.34	504	887	725(100),433(10),271(13)	Pelargonidin-3-p-coumaroylrutinoside-5-glucoside	235 ± 1*	332 ± 1*	nd
6r	35.54	507	917	755(100),433()21,271(41)	Pelargonidin-3-p-feruloylrutinoside-5-glucoside isomer	nd	nd	202 ± 1
7r	36.48	506	917	755(100),463(15),271(38)	Pelargonidin-3-p-feruloylrutinoside-5-glucoside	123.8 ± 0.3[c]	128.9 ± 0.4[b]	202±1[a]
					Total anthocyanin content	**815 ± 2[c]**	**875.6 ± 0.3[b]**	**886 ± 2[a]**

Purple flesh   *Marble*

Peak	Rt (min)	λ_max (nm)	[M]+ (m/z)	MS² (m/z)	Tentative identification	Purple	Violetta	Kefermarkter Blaue	Shetland Black
1p	7.46	530	787	479(100),317(15)	Petunidin-3-rutinoside-5-glucoside	134.3 ± 0.1[a]	50.57 ± 0.01[c]	113.2 ± 0.2[b]	125.9 ± 0.2
2p	10.04	515	771	479(100),317(16)	Petunidin-rutinoside-rhamnoside or petunidin-dirhamnoside-glucoside	nd	43 ± 1	nd	113 ± 1
3p	25.34	525	933	625(100),479(23),317(37)	Positional isomer of peak 5p	nd	43.1 ± 0.4*	104.5 ± 0.2*	102 ± 0.1
4p	27.66	529	949	625(100),479(34),317(46)	Petunidin-3-caffeoylrutinoside-5-glucoside	nd	46 ± 1*	106.5 ± 0.1*	102.0 ± 0.2
5p	33.3	530	933	771(100),479(18),317(22)	Petunidin-3-p-coumaroylrutinoside-5-glucoside	112.3 ± 0.1[c]	175 ± 2[b]	208 ± 3[a]	158.2 ± 0.3
6p	34.69	531	963	625(100),479(10),317(30)	Petunidin-3-feruloylrutinoside-5-glucoside	101.6 ± 0.2[b]	51.7 ± 0.3[c]	113 ± 1[a]	104.1 ± 0.2
7p	36.44	524	947	785(100),463(23),301(38)	Peonidin-3-feruloylrutinoside-5-glucoside	130 ± 1[a]	64.3 ± 0.6[c]	106.8 ± 0.2[b]	117 ± 1
8p	39.13	529	771	625(100),479(14),317(26)	Petunidin-3-p-coumaroylrutinoside	nd	40.3 ± 0.1	nd	nd
					Total anthocyanin content	**478 ± 1[c]**	**513 ± 4[b]**	**533 ± 3[a]**	**481 ± 1**

nd – not detected. Standard Calibration Curve used for quantification: Peonidin-3-*O*-glucoside ($y = 151438x - 3E+06$, $R^2 = 0.9977$, LOD = 0.20 μg/mL and LOQ = 0.71 μg/mL). In each column different Latin letters are significantly different according to Tukey's HSD test ($p=0.05$), for each potato group. *Mean statistical differences obtained by *t*-Student test.

Table 2. *In-vitro* antioxidant and cytotoxicity activity (µg/mL) of aqueous extracts from red, purple and marble (yellow/purple) potato varieties (mean ± SD).

Potato varieties	Antioxidant activity (TBARS) IC$_{50}$ values	Cytotoxicity (PLP2) GI$_{50}$ values
Red flesh		
Rosemary	416.9 ± 4.9[e,f]	>400
Red Emmalie	669.4 ± 4.2[a]	>400
Red Cardinal	591.79 ± 8.1[b]	>400
Purple fleshed		
Purple	426.2 ± 2.6[e]	>400
Violetta	380.1 ± 6.2[f]	>400
Kefermarkter Blaue	484.5 ± 2.1[d]	>400
Marble-fleshed (purple/yellow)		
Shetland Black	547.6 ± 6.4[c]	>400

IC$_{50}$: extract concentration corresponding to 50% of antioxidant activity (against lipid peroxidation). GI$_{50}$: values correspond to the sample concentration achieving 50% of growth inhibition in liver primary culture PLP2; maximum tested concentration: 400 µg/mL. Positive control: Trolox IC$_{50}$ value - 139±5 µg/mL (TBARS); Ellipticine GI$_{50}$ value - 3.2±0.7 µg/mL (PLP2). In each line different Latin letters are significantly different according to Tukey's HSD test (*p*=0.05).

Table 3. Antibacterial and antifungal activities (mg/mL) of aqueous extracts from red, purple and marble-fleshed potato varieties.

Potato Variety	*Bacteria*	S.a.	B.c.	L.m.	E.c.	S.t.	E.c.	*Fungi*	A.f	A.n.	A.v.	P.f.	P.v.c.	T.v.
Red-fleshed														
Rosemary	MIC	4	2	3	3	4	3	MIC	0.5	0.5	0.5	0.35	0.5	0.25
	MBC	8	4	4	4	8	4	MFC	1	1	1	0.5	1	0.5
Red Emmalie	MIC	2	1.5	2	3	3	3	MIC	0.5	0.5	0.5	0.5	1	0.35
	MBC	4	2	4	4	4	4	MFC	1	1	1	1	2	0.5
Red Cardinal	MIC	2	1	2	1.5	3	3	MIC	1	0.5	1	0.5	0.5	0.25
	MBC	4	2	4	2	4	4	MFC	2	1	2	1	1	0.5
Purple-fleshed														
Purple	MIC	3	1	3	1.5	3	3	MIC	0.5	0.5	0.5	0.5	0.5	1
	MBC	4	2	4	2	4	4	MFC	1	1	1	1	1	2
Violetta	MIC	3	1	3	1.5	3	3	MIC	0.25	0.125	1	0.25	0.5	0.125
	MBC	4	2	4	2	4	4	MFC	0.5	0.25	2	0.5	1	0.25
Kefermarkter Blaue	MIC	6	3	3	3	6	6	MIC	0.25	0.125	0.35	0.25	0.5	0.125
	MBC	8	4	4	4	8	8	MFC	0.5	0.25	0.5	0.5	1	0.25
Marble-fleshed														
Shetland Black	MIC	4	1	3	1	4	4	MIC	1	0.5	0.5	0.75	0.5	0.25
	MBC	8	2	4	2	8	8	MFC	2	1	1	1	1	0.5
Controls														
E211	MIC	4.0	0.5	1.0	1.0	1.0	2.0	MIC	1.0	1.0	2.0	1.0	2.0	1.0
	MBC	4.0	0.5	2.0	2.0	2.0	4.0	MFC	2.0	2.0	2.0	2.0	4.0	2.0
E224	MIC	1.0	2.0	0.5	0.5	1.0	0.5	MIC	1.0	1.0	1.0	0.5	1.0	0.5
	MBC	1.0	4.0	1.0	1.0	1.0	0.5	MFC	1.0	1.0	1.0	0.5	1.0	0.5

MIC: minimal inhibitory concentration (mg/mL); MBC: minimal bactericidal concentration (mg/mL); and MFC: minimal fungicidal concentration (mg/mL). Bacterial strains: S.a. (*Staphylococcus aureus*; ATCC 6538*)*, B.c.. (*Bacillus cereus*; food isolate*)*, L.m. (*Listeria monocytogenes*; NCTC 7973), E.c. (*Escherichia coli*; ATCC 35210*)*, S.t. (*Salmonella* Typhimurium; ATCC 13311), and En.cl. (*Enterobacter cloacae*; human isolate). Fungal strains: *A.f. (Aspergillus fumigatus,* human isolate)*;* A.n. (*Aspergillus niger*; ATCC 6275), A.v. (*Aspergillus versicolor*; ATCC 11730), P.f. (*Penicillium funiculosum*; ATCC 36839), P.a. (*Penicillium aurantiogriseum)*; food isolate), T.v. (*Trichoderma viride*; IAM 5061). Positive controls: E211- sodium benzoate and E224 - potassium metabisulphite.

Figure 1. Fresh potato tubers of the genotypes: 1 – Rosemary; 2 - Red Emmalie; 3 - Red Cardinal; 4 – Purple; 5 – Violetta; 6 - Kefermarkter Blaue; 7 – Shetland Black, and the main class of anthocyanidins found in a) red-fleshed varieties and b) purple-fleshed varieties.
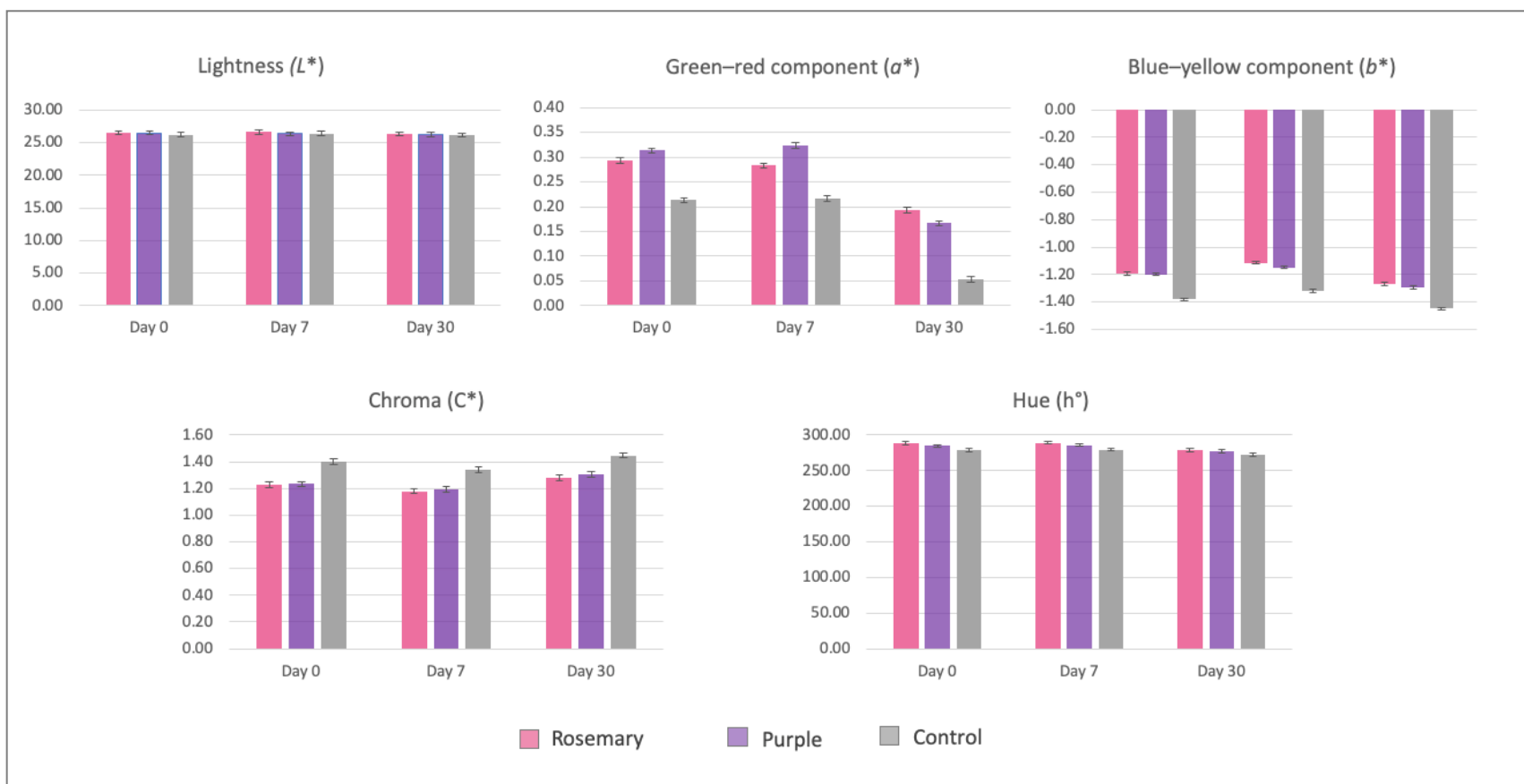
Figure 2. CIELab colour values (*L**, *a** and *b**) and cylindrical coordinates (C* and h°) of the soft drink formulations *Rosemary* (red potato extract), *Purple* (purple potato extract) and *Control* (E163 commercial colouring), over a 30 days shelf-life period. L*: lightness from black (0) to white (100); *a**: green (−) to red (+), *b**: blue (−) to yellow (+); C*: chroma, relative saturation; and h°: angle of the hue in the CIELab colour space.
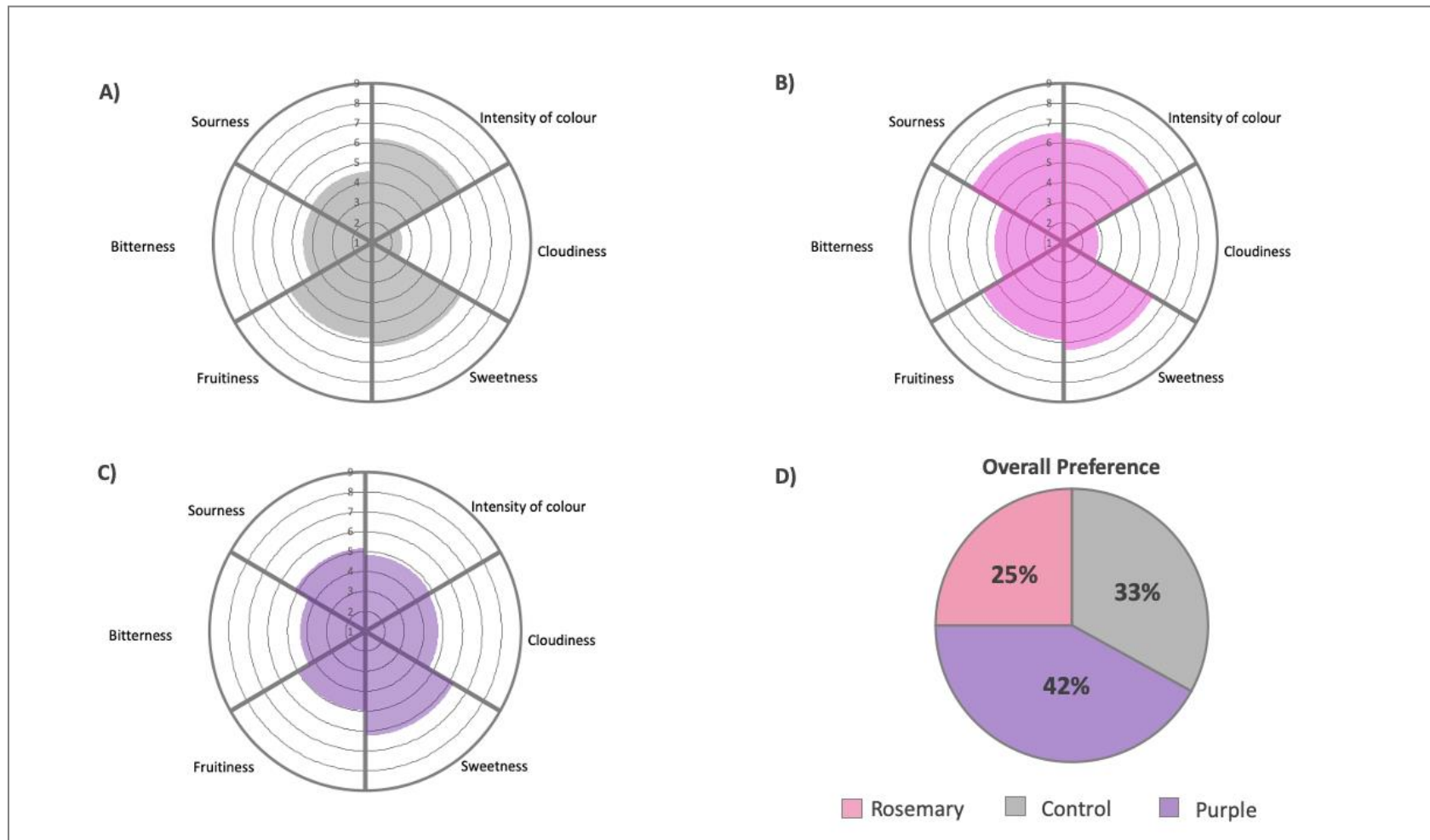
Figure 3. Sensory analysis scores (1 to 9) for the attributes: intensity of colour, cloudiness, sweetness, fruitiness, bitterness and sourness, and overall preference of the tested soft drink formulations. A) *Rosemary* potato extract; B) *Control* E163; C) *Purple* potato extract; D) overall preference scores.