



Article

Buckwheat, Fava Bean and Hemp Flours Fortified with Anthocyanins and Other Bioactive Phytochemicals as Sustainable Ingredients for Functional Food Development

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Abstract: Facing a climate emergency and an increasingly unhealthy population, functional foods should not only address health issues but must be prepared from sustainable ingredients while contributing to our sustainable development goals, such as tackling waste and promoting a healthy environment. High-protein crop flours, i.e., buckwheat, hemp and fava bean, are investigated as potential matrices to be fortified with key bioactive phytochemicals from soft fruits to explore potential waste valorization and to deliver sustainable functional food ingredients. Hemp flour provided the best matrix for anthocyanin fortification, adsorbing of $88.45 \pm 0.88\%$ anthocyanins and 69.77 mg/kg of additional phytochemicals. Buckwheat and fava bean absorbed $78.64 \pm 3.15\%$ and $50.46 \pm 2.94\%$ of anthocyanins 118.22 mg/kg and 103.88 mg/kg of additional phytochemicals, respectively. During the fortification, there was no detectable adsorption of the berry sugars to the flours, and the quantities of free sugars from the flours were also removed. One gram of fortified hemp flour provides the same amount of anthocyanins found in 20 g of fresh bilberries but has substantially less sugar. The optimum conditions for high protein flour fortification with anthocyanins was established and showed that it is a viable way to reduce and valorize potential agricultural waste, contributing to a circular and greener nutrition.

Keywords: buckwheat; fava bean; hemp; anthocyanin; flour fortification; functional food; berries; soft fruit; agricultural waste; bilberry



Citation: Neacsu, M.; Christie, J.S.; Duncan, G.J.; Vaughan, N.J.; Russell, W.R. Buckwheat, Fava Bean and Hemp Flours Fortified with Anthocyanins and Other Bioactive Phytochemicals as Sustainable Ingredients for Functional Food Development. *Nutraceuticals* **2022**, *2*, 150–161. <https://doi.org/10.3390/nutraceuticals2030011>

Academic Editors: Ivan Cruz-Chamorro and Luisa Tesoriere

Received: 13 May 2022

Accepted: 7 July 2022

Published: 14 July 2022

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1. Introduction

Soft fruits are particularly prone to wastage and, in the case of strawberries alone, one in ten ends up as waste in the UK [1]. In 2015 alone, just over 9% of mature strawberries ended up as waste, as the product did not meet quality requirements, primarily because of fruit being misshapen [1]. It is essential that UK producers find higher value and novel uses for soft fruit waste, ultimately contributing to food waste reduction and sustainable food production. Anthocyanins are a major group of phytochemicals from soft fruits, shown to inhibit carbohydrate uptake from food, resulting in decreased postprandial blood glucose [2] and the inhibition of endogenous enzymes, including amylase, sucrase [3] and glucosidases [4], all enzymes associated with carbohydrate digestion. Supplementation with bilberry extract has been shown to modify the glycemic response in volunteers with type 2 diabetes mellitus (T2DM) [5], and wild bilberry-fortified soya flour reduced post prandial blood glucose levels in mice, along with weight gain [2]. As obesity and sustained high blood sugar levels are major risk factors in the development of T2DM [6], it is likely that products rich in dietary anthocyanin could contribute towards decreasing the incidence of this condition, delivering economically desirable preventative nutritional therapies. Revalorizing soft fruit bioactives and reintroducing them back into foods could

deliver solutions to aid metabolically compromised individual and help reduce soft fruit agricultural waste.

High-protein crops, such as hemp, buckwheat and fava bean, besides having the potential to be grown sustainably in certain climates, could constitute key ingredients of a healthy, sustainable diet. We have shown that hemp and buckwheat flours are valuable sources of dietary amino acids, beneficially modulating gastrointestinal hormones and promoting satiety in healthy volunteers [7]. Foods prepared from buckwheat showed health benefits, attenuating insulin resistance and improving lipid profiles in patients with type 2 diabetes [8]. Buckwheat contains proanthocyanidins, which can inhibit digestive enzymes [9] and D-chiro-inositol, which can function as a mediator for anti-hyperinsulinemia [10,11]. Hemp flour is also a rich source of dietary fiber, around 25% [12], which could exert a prebiotic effect [13] and lower blood cholesterol [14].

Modern functional foods should not only provide a proven health benefit but should contribute to our sustainable development goals by finding solutions to tackle agricultural waste and contribute to a healthy and green environment. In the present paper, buckwheat, hemp and fava bean flours are investigated for their capacity to adsorb bioactive phytochemicals from bilberries, establishing the optimum conditions to enrich the flours with anthocyanins, while retaining no detectable sugar content from the soft fruits. This anthocyanin-rich food could represent a viable candidate to be incorporated into nutritional therapies for T2DM management. While the main aim of the work is to deliver the development of a functional food ingredient concept, this methodology could also be used to provide alternative solutions to tackle fruit and plant-based waste in general by revalorizing bioactive phytochemicals (anthocyanin). This work discusses innovative, climate-friendly, healthy and sustainable food ingredients to meet consumers' needs, while could also be addressing critical issues regarding agricultural waste.

2. Materials and Methods

Plant materials: Wild bilberries (*Vaccinium* sp.) were picked from the Tyrebagger Forest in Aberdeen, Scotland and stored at -80°C . The berries were freeze dried (Labconco; Kansas City, MO, USA), freeze milled (Spex sample prep 6800; Munich, Germany) and stored at room temperature in desiccators under vacuum with exclusion of light. The hemp flour was purchased from Yorkshire Hemp (Driffield, UK), fava bean flour from Barry Farm (Cridersville, OH, USA) and buckwheat flour from Arrowhead Mills (Boulder, CO, USA). All flours were stored at room temperature with the exclusion of light, following the manufacturer's recommendation storage conditions.

Standards and reagents: Standards for the anthocyanin aglycones (anthocyanins), delphinidin (>95%), cyanidin (>95%), pelargonidin (undeclared purity), peonidin (>96.5%), were all purchased from Sigma-Aldrich (Dorset, UK) and malvidin (>95%) from Phytolab, Germany. The aglycone standard, petunidin, was purchased from ChemFaces (Hubei, China) at a purity of >95%. The anthocyanin glycoside (anthocyanidin) standards; delphinidin 3-glucoside (>97%), cyanidin 3-glucoside (>95%), cyanidin 3-galactoside (>95%), petunidin 3-glucoside (>95%), peonidin 3-glucoside (>95%), pelargonidin 3-glucoside (>97%) and malvidin 3-glucoside (>95%), were all purchased from Sigma-Aldrich (Dorset, UK). All the phenolic standards were purchased from Sigma-Aldrich (Gillingham, UK), Phytolab, Germany or synthesized as described previously [15,16]. General reagents were purchased from Sigma-Aldrich (Dorset, UK) and Fischer Scientific (Loughborough, UK).

A schematic summary of the succession of procedures used in the experimental protocol is presented in the Figure 1.

Flour fortification with wild bilberry phytochemicals: The freeze-dried wild bilberries were used as a source of anthocyanins and other phenolics to enrich the buckwheat, fava bean and hemp flour. The freeze-dried berry powders were suspended at three different concentrations of 0.1 (A), 0.07 (B) and 0.04 g/mL (C), in citric acid (4.75 mM, pH 3.5). Suspensions were placed in an ultrasound bath for 5 min and then intermittently mixed at room temperature for 1 h. The supernatant was separated by centrifugation (5 min;

3220 × g; 4 °C). This extract was used for the flour fortification experiments. Three different quantities of each flour, 1 g, 0.5 g and 0.2 g, respectively (n = 3) were suspended in the extracts prepared above (5 mL). The suspensions were then thoroughly mixed at room temperature for 5 min. The supernatants were separated by centrifugation (5 min; 3220 × g; 4 °C), filtered (0.2 μm) and immediately analyzed.

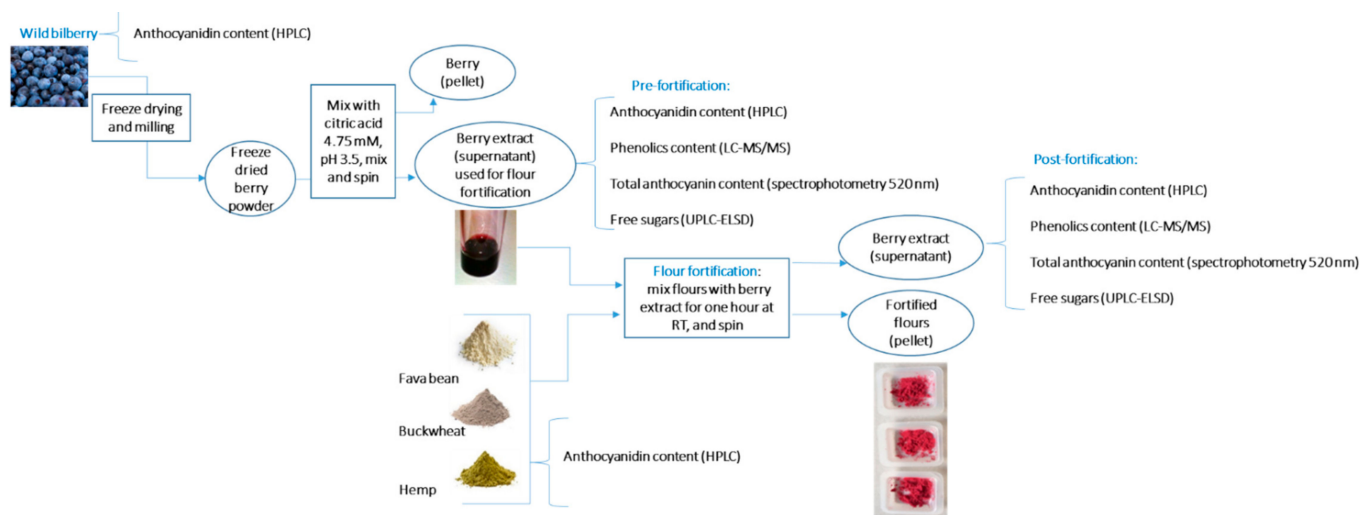


Figure 1. Overview of the experimental procedures used for the fortification of buckwheat, fava bean and hemp flours, highlighting the measurements (and methodologies) used in the experimental protocol.

The flour fortification (the amounts of anthocyanins and other phenolics added to the flour) was measured by comparing the total anthocyanin content (measured by UV-VIS spectrophotometry), the individual anthocyanins (measured by HPLC-DAD), other phenolics (measured by LC-MS/MS) and sugar content (measured by UPLC-ELSD) in the wild bilberry extracts (pre-fortification) and the resulting supernatants (post-fortification).

Estimation of total anthocyanins adsorbed on the flours: Total anthocyanin content was estimated by measuring the absorption between 400 nm and 700 nm for the extracts (pre-fortification) and resultant supernatants (post-fortification) using a μ Quant 7271000 plate reader Biotek Instrument (Potton, UK). Subsequent absorption measurements were made at the λ_{max} (520 nm). The semi-quantification for total anthocyanin glycoside content was performed using the cyanidin 3-glucoside standard.

Determination of individual anthocyanidin content from the bilberries, buckwheat, fava bean, hemp flours and anthocyanin adsorbed on the flours: To measure the anthocyanidin content of the bilberries and flours was used the extraction and hydrolysis methods adapted from [17]. Briefly, samples (n = 3) of wild bilberry (0.05 g) and buckwheat, fava bean and hemp flour (1 g) were extracted with methanol:water:hydrochloric acid (ratio of 50:33:17; v/v/v; 3 mL) three times, and the supernatants and the pellet combined and hydrolyzed at 100 °C for 60 min. Hydrolyzed samples were then immediately cooled to room temperature, filtered using 0.2 μm filters and analyzed by HPLC.

The quantification of the anthocyanins and anthocyanidins was performed using a 1260 Infinity HPLC from Agilent (Wokingham, UK) and a Synergi 4 μm Polar-RP 80A (250 × 4.6 mm) column with a Polar-RP 4 × 3 mm pre-column from Phenomenex (Macclesfield, UK). The DAD spectra were recorded between 200 and 700 nm and the chromatograms were monitored at 530 nm for the detection of the anthocyanidins and 520 nm for the glycoside forms (anthocyanins). For the HPLC separations the following solvents were used: A: formic acid (2.125%) and B: acetonitrile/methanol (85:15, v/v).

The HPLC method for anthocyanidin analysis was adapted from Zhang, Z. et al., (2004) [17]. The solvent program was isocratic 18% B for 40 min. The flow was constant at 1 mL/min and the column temperature was held at 35 °C. The anthocyanins content

from the fortification extracts was determined directly in the extracts as prepared above for fortification using the HPLC method analysis adapted from [18]. The column temperature was constant at 28 °C using a solvent and flow rate gradient program.

The separation and quantification of anthocyanins was performed using external standardization with delphinidin ($r^2 = 0.998$, LOD = 8.52 µg/mL), cyanidin ($r^2 = 0.997$, LOD = 11.31 µg/mL), petunidin ($r^2 = 0.999$, LOD = 5.64 µg/mL), pelargonidin ($r^2 = 0.997$, LOD = 12.63 µg/mL), peonidin ($r^2 = 0.999$, LOD = 10.57 µg/mL), malvidin ($r^2 = 0.99$, LOD = 6.09 µg/mL), delphinidin 3-glucoside ($r^2 = 0.997$, LOD = 14.78 µg/mL), cyanidin 3-glucoside ($r^2 = 0.994$, LOD = 35.76 µg/mL), cyanidin 3-galactoside ($r^2 = 0.997$, LOD = 19.89 µg/mL), peonidin 3-glucoside ($r^2 = 0.999$, LOD = 2.36 µg/mL), pelargonidin 3-glucoside ($r^2 = 0.999$, LOD = 3.05 µg/mL) and malvidin 3-glucoside ($r^2 = 0.999$, LOD = 3.00 µg/mL).

LC-MS/MS analysis of other phenolics adsorbed on the flours: The wild bilberry aqueous extracts (0.1 g/mL) and the solution obtained after the flour fortification (0.04 g flour to each mL extract as described above) were collected and freeze dried. Freeze-dried samples (approx. 0.1 g dry weight; $n = 3$) were suspended in hydrochloric acid (2 M; 3 mL) and incubated at 90 °C for one hour with intermittent mixing. They were then cooled to room temperature and extracted with ethyl acetate three times, the organic layers were combined, filtered through sodium sulphate (anhydrous), the solvent was evaporated and the residue was dissolved in methanol/water (50:50, v/v ; 0.5 mL) for LC-MS/MS analysis using internal standard for negative mode mass spectrometry ^{13}C benzoic acid at 2 µg/mL and, respectively, 2-amino-3,4,7,8-tetramethylimidazo(4,5-f)quinoxaline at 0.5 µg/mL, for positive mode mass spectrometry.

The LC-MS/MS analysis methods used for phenolics analysis was performed as previously published [19–21] and quantified using multiple reaction monitoring and internal standardization. The liquid chromatography separation of the metabolites was performed on an Agilent 1100 LC-MS system (Agilent Technologies, Wokingham, UK) using a Zorbax Eclipse 5 µm, 150 mm × 4.6 mm C18 column (Agilent Technologies). For all the phenolics quantifications the standard calibrations were over a concentration interval of 2 µg/mL to 10 ng/mL. The threshold used for quantification was a signal to noise ratio of 3 to 1. All the ion transitions for each of the metabolites were determined based upon their molecular ion and a strong fragment ion and their voltage parameters; their declustering potential, collision energy and cell entrance/exit potentials were optimized individually for each metabolite and have been previously described [18,19].

UPLC-ELSD quantification of mono- and disaccharides from wild bilberry fortification extracts: The separation and quantification of sugars used external standardization with fructose ($r^2 = 0.999$, LOD = 0.65 mg/mL), glucose ($r^2 = 0.997$, LOD = 0.70 mg/mL) and sucrose ($r^2 = 0.996$, LOD = 0.41 mg/mL). The instrument used was a Waters Acquity UPLC (Elstree, UK) equipped with an Acquity UPLC BEH Amide column (Elstree, UK) (1.7 µm 2.1 × 100 mm). Gradient elution starting with 100% mobile phase A (80% acetonitrile in water with 0.2% triethylamine), decreasing to 60% A and 40% B (30% acetonitrile in water with 0.2% triethylamine) over 10 min, followed by a 30-min isocratic elution at 100% A. Flow rate and temperature were constant at 0.12 mL/min and 23 °C, respectively. The ELSD detector used a gain of 200 and pressure at 40 psi.

Statistical analysis: All the data were averaged from three technical replicates of samples and are reported as means and standard deviation. The differences between concentrations of various macronutrients and phytochemicals between various plant materials from this study was assessed by two-sided post hoc *t*-tests, with *p* values less than 0.05 ($p < 0.05$) indicating significance. Microsoft® Excel® for Office 365 (Microsoft Corporation, Redmond, WA, USA) was used for statistical analyses.

3. Results

3.1. Anthocyanidin Content of Wild Bilberry, Buckwheat, Fava Bean and Hemp Flours

Bilberry samples were found to be rich in five of the six anthocyanidins measured; delphinidin (10.87 ± 1.57 g/kg dry weight), cyanidin (14.13 ± 1.47 g/kg dry weight), petunidin (3.18 ± 0.29 g/kg dry weight), peonidin (2.8 ± 0.21 g/kg dry weight) and malvidin (34.33 ± 2.94 g/kg dry weight). Pelargonidin was the only aglycone that was not detected. Cyanidin and pelargonidin were detected in buckwheat flour with concentrations of 170.83 ± 9.16 and 227.43 ± 11.01 mg/kg dry weight, respectively. Delphinidin and cyanidin were detected in fava bean flour at concentrations of 4.1 ± 0.31 and 2.23 ± 0.23 mg/kg dry weight, respectively. Cyanidin was the only anthocyanidin detected in hemp flour at a concentration of 5.77 ± 1.72 mg/kg.

3.2. Bilberry Anthocyanins Adsorbed by the Buckwheat, Fava Bean and Hemp Flour

The optimum conditions for fortifications (defined as highest anthocyanins quantity adsorbed by a minimum amount of flour) were 0.1 g/mL freeze-dried wild bilberry at a ratio of flour to extract of 0.04 g flour to each mL extract. By increasing the concentration and volume of the aqueous extract, the anthocyanin fortification of the flours was increased with hemp flour adsorbing the highest quantities of bilberry anthocyanins. This translates to 95.69 ± 12.47 mg of total anthocyanins per g of buckwheat flour, representing $78.64 \pm 3.15\%$ anthocyanins removed from the extracts or attached to the flour (Table 1); 88.86 ± 8.44 mg of total anthocyanins per g of fava bean flour ($50.46 \pm 2.94\%$ anthocyanins removed from the bilberry extract) and 101.64 ± 1.57 of total anthocyanins per g of hemp flour ($88.45 \pm 0.88\%$ anthocyanins removed from the bilberry extract), as can be seen in Table 1. This paper's results are in agreement with other researchers' work where high protein flours, such as soybean, adsorbed anthocyanins in a similar range of concentrations and amounts [22].

Table 1. Total berry anthocyanins (mg of total anthocyanins per g of flour) adsorbed by the buckwheat, fava bean and hemp flour.

Flour	Aqueous Wild Bilberry Extracts		
	A *	B *	C *
Buckwheat			
0.2 g/mL extract	24.93 ± 0.72 (97.02 ± 0.69)	10.07 ± 0.55 (94.56 ± 1.98)	5.26 ± 0.87 (92.91 ± 1.53)
0.1 g/mL extract	48.11 ± 2.19 (92.93 ± 1.6)	19.5 ± 1.08 (92 ± 1.76)	10.31 ± 1.67 (91 ± 1.38)
0.04 g/mL extract	95.69 ± 12.47 (78.64 ± 3.15)	26.23 ± 4.39 (48.64 ± 8.69)	14.27 ± 4.6 (8.56 ± 5.14)
Fava bean			
0.2 g/mL extract	24.43 ± 3.46 (91.26 ± 0.34)	10.76 ± 0.43 (85.04 ± 0.18)	12.91 ± 0.99 (90.85 ± 0.4)
0.1 g/mL extract	41.68 ± 6.51 (76.69 ± 2.9)	19.32 ± 0.66 (75.97 ± 1.05)	25.4 ± 1.99 (88.67 ± 0.66)
0.04 g/mL extract	88.86 ± 8.44 (50.46 ± 2.94)	24 ± 6.73 (37.02 ± 8.93)	31.58 ± 4.54 (52.55 ± 2.02)
Hemp			
0.2 g/mL extract	23.05 ± 0.95 (94.33 ± 0.4)	8.66 ± 0.89 (90.72 ± 1.01)	7.04 ± 0.4 (92.1 ± 0.93)
0.1 g/mL extract	44.92 ± 2.1 (91.83 ± 0.28)	16.48 ± 1.7 (86.67 ± 1.07)	14.07 ± 0.89 (91.6 ± 1.25)
0.04 g/mL extract	101.64 ± 1.57 (88.45 ± 0.88)	36.29 ± 6.28 (78.95 ± 3.61)	20.2 ± 0.62 (60.75 ± 0.65)

Values given are mean \pm standard deviation ($n = 3$) in mg of total anthocyanins per g of flour. The values in parenthesis represent total anthocyanin quantities (%) removed from the extracts or attached to the flours. Total anthocyanins quantities were determined spectrophotometrically, the values are expressed in cyanidin 3-glucoside relative units. * Represents concentrations of bilberry extracts in aqueous solution at pH 3.5 citric acid prepared by suspending freeze-dried wild bilberry powder at concentrations of 0.1 g/mL (A), 0.07 g/mL (B) and 0.04 g/mL (C).

The decrease in the mass of flour per volume of juice increased the mass of anthocyanins ($p < 0.05$) bound to the flour. This increase in juice to flour ratio led to less efficient removal of polyphenols from juices. However, the main purpose of this work

this is the enrichment of the flours and, for commercial applications, the non-absorbed polyphenols could represent a considerable expense. Similarly, increasing the concentration of the bilberry juice shows significant increases in the level of enrichment ($p < 0.01$) of each flour without affecting the level of non-absorbed anthocyanins. It is possible that further increases in concentration or volume could further increase the quality of enrichment but given the non-absorbed polyphenols already seen, this may not be practical or economically viable. The optimum condition of anthocyanin enrichment for all three flours with bilberry extracts was by suspending 0.04 g flour in 1 mL juice of concentration A (0.1 g/mL bilberry powder in citric acid pH 3.5).

The profile of the individual anthocyanins before and after fortification, using the optimum fortification conditions, was quantified by HPLC-DAD and are presented in Figure 2. The results showed a significant ($p < 0.001$) fortification with the major anthocyanins, identified as delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside. It appeared that several additional anthocyanin glycosides were also observed in the HPLC chromatogram, but these could not be identified, as standards were not available. In all the cases, the fortification efficiency was higher for delphinidin-3-glucoside and cyanidin-3-galactoside at concentrations of $64.52 \pm 1.57\%$ and $65.55 \pm 0.74\%$ for buckwheat, $55.25 \pm 0.33\%$ and $50.25 \pm 0.35\%$ for fava bean and $68.29 \pm 2.31\%$ and $71.06 \pm 0.79\%$ for hemp flour, respectively (Table 2). Malvidin-3-glucoside was least efficiently adsorbed by the flours at concentrations of $46.94 \pm 0.44\%$, $33.93 \pm 0.42\%$ and $53.83 \pm 0.9\%$ for buckwheat, fava bean and hemp flour, respectively (Table 2).

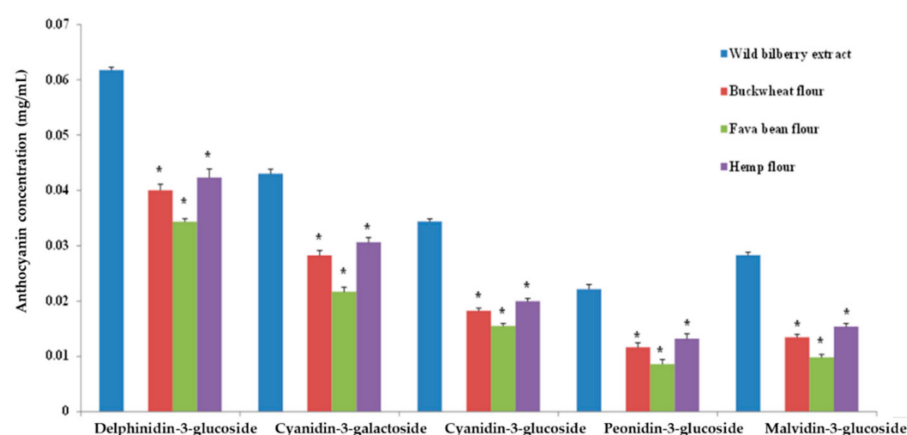


Figure 2. The adsorption of anthocyanin on the flours, as indicated by the amount (mg/mL) removed from aqueous wild bilberry extract (0.1 g/mL) with citric acid (pH 3.5). The first bar in each set (blue color) represents the concentration of individual anthocyanin in the initial wild bilberry extract used for the fortification of the flours. The subsequent bars represent individual concentration of anthocyanin adsorbed on each of the flours after fortification. For fortification, 0.04 g of flour was used for each mL of berry extract, where (*) represents significant reduction in the extract concentration of individual anthocyanin (or adsorption of individual anthocyanin on the flours from the extract) after the flour fortification ($p > 0.0001$).

Table 2. Individual anthocyanin adsorption (%) on the flours from wild bilberry extracts.

Flour	Flours Anthocyanins Fortification (%)				
	Delphinidin-3-Glucoside	Cyanidin-3-Glucoside	Cyanidin-3-Galactoside	Peonidin-3-Glucoside	Malvidin-3-Glucoside
Buckwheat	64.52 ± 1.57	65.55 ± 0.74	52.65 ± 0.2	51.53 ± 0.51	46.94 ± 0.44
Fava bean	55.25 ± 0.33	50.25 ± 0.35	44.53 ± 0.3	37.46 ± 0.51	33.93 ± 0.42
Hemp	68.29 ± 2.31	71.06 ± 0.79	57.68 ± 0.78	58.62 ± 1.51	53.83 ± 0.9

Values given are mean \pm standard deviation ($n = 3$) as % of individual anthocyanins adsorbed on the flours from the wild bilberry extracts. Flour fortification was performed using aqueous wild bilberry extract (0.1 g/mL). For each mL of extract, 0.04 g flour was used for the fortification.

3.3. Other Phenolics from Wild Bilberries Adsorbed on the Flours

The quantity of several phenolics adsorbed by the flours was calculated by the difference between the amount in the wild bilberry extract before and after the fortification (Figure 3). The wild bilberry extract was found to be rich in phenolic acids including chlorogenic (40.03 ± 7.47 mg/kg), caffeic (28.76 ± 5.51 mg/kg), p-coumaric (28.23 ± 6.18 mg/kg), protocatechuic (11.38 ± 2.63 mg/kg), and vanillic acid (7.83 ± 1.64 mg/kg); and in flavonoids; quercetin (31.52 ± 5.31 mg/kg), myricetin (20.09 ± 4.6 mg/kg), and isorhamnetin (3.97 ± 0.94 mg/kg), (Figure 3).

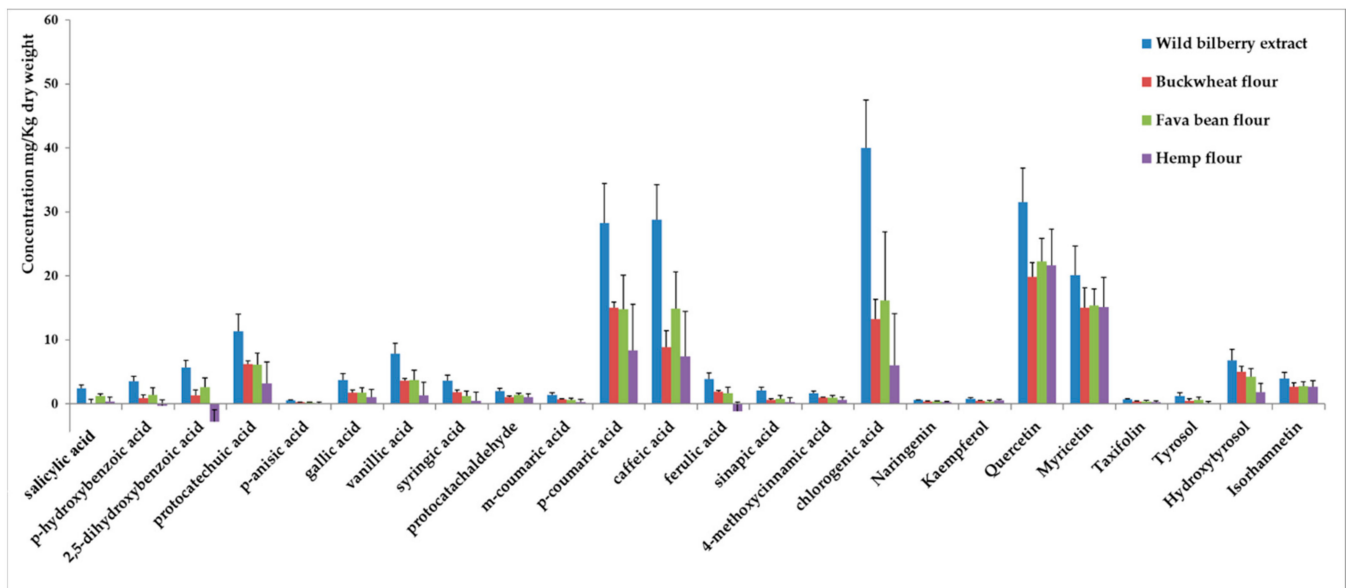


Figure 3. The main phenolics quantified in the initial aqueous wild bilberry extract (0.1 g/mL prepared in citric acid pH 3.5) used for the flour fortification (blue bars) and the adsorption of individual phenolic molecules (mg/kg dry weight) from the initial extract on the buckwheat flour (red bars) on fava bean flour (green bars) and hemp flour (purple bars). For the fortification, 0.04 g from each flour for each mL of wild bilberry extract was used.

From the flavonoids present in the initial extract used for the flour fortification, myricetin had the best adsorption on the fava bean flour $77.68 \pm 12.6\%$, (corresponding to 15.07 ± 3.09 mg/kg dry weight from the initial extract), followed by isorhamnetin and quercetin with $71.93 \pm 15.16\%$ and $71.85 \pm 15.97\%$ adsorbed from the initial extract (corresponding to 2.82 ± 0.64 mg/kg and 22.27 ± 3.60 mg/kg dry weight from the initial extract), respectively (Figure 3). From the phenolic acids present in the initial extract used for flour fortification, protocatechuic acid had the best adsorption on the fava bean flour with $56.75 \pm 24.52\%$, (corresponding to 6.12 ± 1.84 mg/kg dry weight from initial extract) being adsorbed. This was followed by p-coumaric and caffeic acid with $55.37 \pm 26.95\%$ and $54.54 \pm 27.36\%$ being adsorbed from the initial extract (corresponding to 14.77 ± 5.34 mg/kg and 17.87 ± 5.74 mg/kg dry weight from initial extract), respectively. Chlorogenic acid, which was the richest by weight on the fortified fava bean flour with 16.18 ± 10.71 mg/kg dry weight from the initial extract being adsorbed, representing $42.60 \pm 30.60\%$ adsorbed on the buckwheat flour, 78.61% on fava bean flour and 75.37% on hemp flour (Figure 3). Some phenolic acids, such as 2,3-dihydroxybenzoic acid in buckwheat and p-hydroxybenzoic, 2,5-dihydroxybenzoic and ferulic acid in hemp, were released from the flours into extracts during the fortification process (Figure 3). Overall, the flavonoids were better adsorbed compared to the phenolic acids, and from approximately 218 mg/kg total phenolics measured in the wild bilberry extract, 118 mg, 103 mg and 70 mg were adsorbed by the fava bean, buckwheat and hemp flours, respectively.

3.4. Sugar Content Following the Flour Fortification with Wild Bilberry

The free sugar content of the aqueous wild bilberry extracts (pre-fortification) and the remaining supernatants (post-fortification) were determined using UPLC-ELSD (Figure 4). None of the flours retained any of the sugars present in the aqueous bilberry extracts and the important quantities of the sugars were extracted from the flours during the fortification process (Figure 4). The total sugar content (representing the sum of fructose, glucose and sucrose) was measured before and after flour fortification. Comparing the initial extract before fortification with the extract recovered after the flour fortification, there was an increase in total sugar content varying from as little as 2.1% for the buckwheat fortification of 0.04 g flour to 1 mL of Extract B up to 55% after hemp fortification of 0.2 g flour with 1 mL of Extract B (prepared by adding 0.07 g wild bilberry powder to 1 mL citric acid pH 3.5).

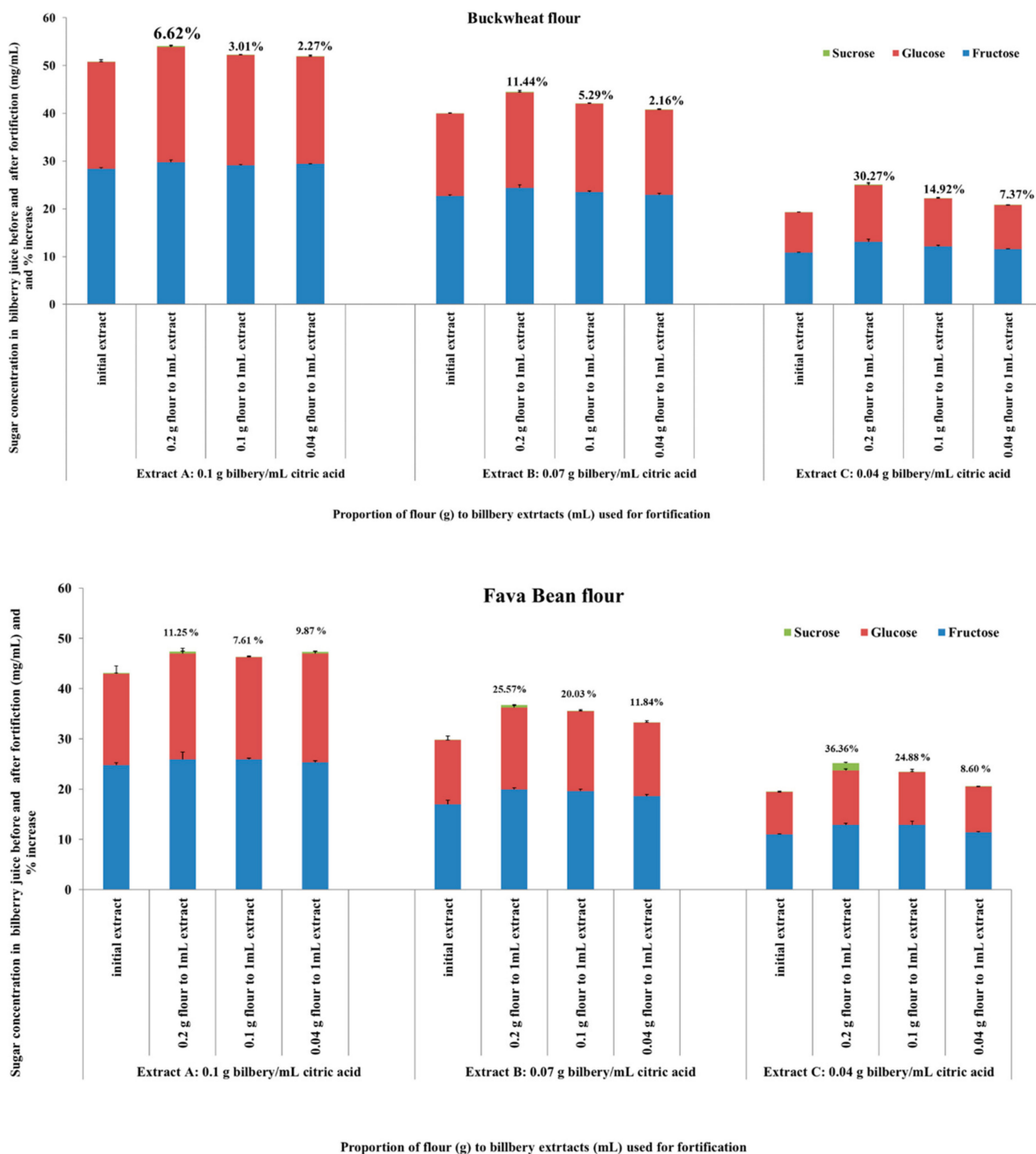


Figure 4. Cont.

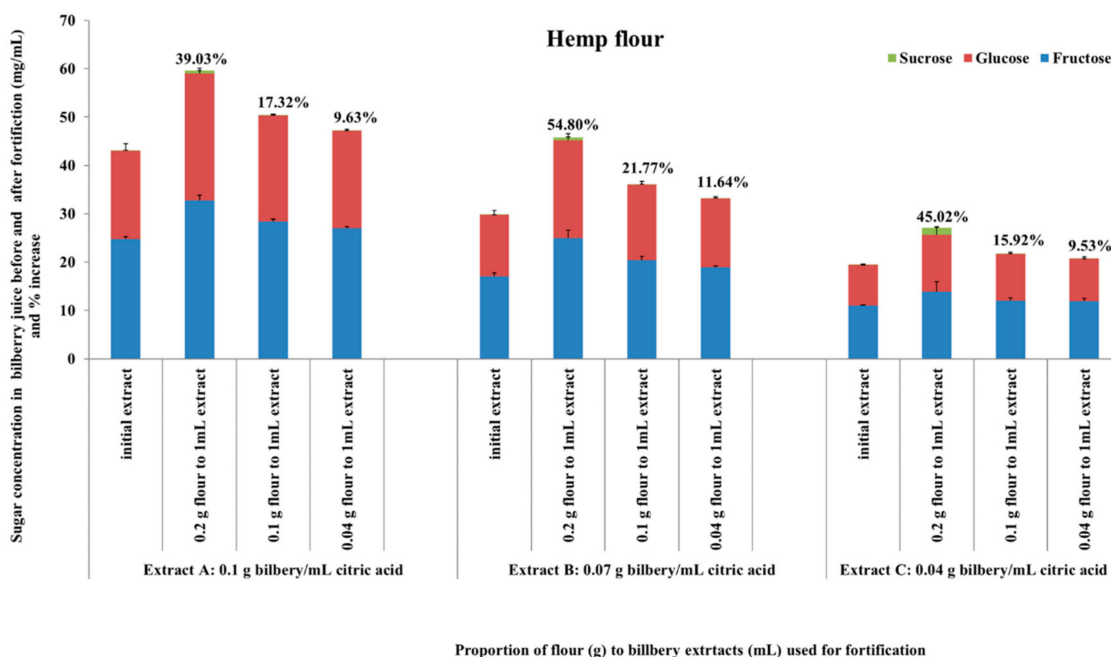


Figure 4. The sugar (glucose, sucrose and fructose) content of the bilberry extracts prepared at concentrations of 0.1 g/mL (Extract A), 0.07 g/mL (Extract B) and 0.04 g/mL (Extract C) before (initial extract) and after flour fortification, using flour to extract ratio of 1 to 5 (0.2 g flour to 1 mL extract), 1 to 10 (0.1 g flour to 1 mL extract) and 1 to 25 (0.04 g flour to 1 mL extract) for each concentration of extract presented above, respectively. The values in the top of each chart represent the total sugar increment (in %) from the initial bilberry extract after the flour fortification.

4. Discussion

As buckwheat, fava bean and hemp fortified flours retained the characteristic color of anthocyanins, it is likely that they are adsorbed, rather than covalently bound. In this context, it could be hypothesized that the compounds present in the flours act as co-pigments for bilberry-derived anthocyanins. It has been shown that most common co-pigments are flavonoids, phenolic acids, alkaloids, amino acids and organic acids [23]. Our previous research [7,12] reported buckwheat, fava bean and hemp flours as rich sources of bound phenolics acids, such as sinapic acid, ferulic acid, caffeic acid and amino acids, including proline and arginine, which are all known efficient co-pigments [23–25]. Furthermore, the optimum conditions used for the flour fortification (pH 3.5 aqueous solution) are also favorable for co-pigmentation [23,26,27].

Using the optimized conditions for fortification, hemp adsorbed the highest quantities of anthocyanins from wild bilberries. Hemp was the best matrix for fortification with each gram of the fortified hemp flour potentially delivering the equivalent of anthocyanins present in 20 g of fresh berries. This is particularly important considering that anthocyanins have been extensively studied for their health benefits, including the prevention of cardiovascular disease [28–30], anti-cancer properties [31–33] and to benefit people living with type 2 diabetes mellitus [34–36]. Furthermore, buckwheat and hemp flours beneficially modulated gastrointestinal hormones and promoted satiety in healthy volunteers [7]. Therefore, these fortified flours could represent attractive functional foods for prevention and aid of several non-communicable disorders and for weight management in nutritional therapies. The additional phenolics adsorbed during the fortification, including phenolic acids (caffeic, p-coumaric and chlorogenic) and flavonoids (quercetin and myricetin) potentially confer additional health benefits to the final product, complementing to the nutritional value, shelf life and food reformulation versatility.

From 2.5 kg of dried bilberries, 1.88 kg of dried extract for flour fortification can be produced. Considering that 57% of bilberry extract is used for buckwheat fortification, 49% for fava bean fortification and 54% for hemp fortification, the resulting extract could be reused for further flour fortification, delivering a more economically viable process. Moreover, all the conditions and materials used in this process, such as water, citric acid, bilberry fruits, buckwheat, fava bean and hemp, are very accessible and could easily be scaled-up for commercial production.

Functional food ingredients rich in anthocyanins (and, importantly, with no free sugars) could represent a key component in the nutritional therapies for T2DM management for people at risk developing T2DM and people living with the disease. This due to the beneficial health attributes related to the regulation of sugar metabolism, as presented earlier in this paper. Moreover, there is strong scientific evidence from animal and human clinical studies describing the beneficial impact of anthocyanins on cardiovascular and neurodegenerative diseases, as has been recently reviewed by Mattioli et al. [34]. Additionally, the anthocyanin-rich ingredients as food components could also be beneficial to the food itself; it can protect the food from damage caused during baking, while improving its antioxidant capacity, this being superior to even synthetic additives [37]. Moreover, in products such as kefir, yoghurt, and other beverages, anthocyanins have been found to have high stability during storage and improve the color of processed foods, representing a viable alternative to synthetic colorants as highlighted in a recent review on food product fortification with anthocyanins carried out by Echeagaray et al. [37].

Therefore, the health and food applications of anthocyanins-rich ingredients could be numerous, and the projections of the functional food market are very healthy; they were valued at USD 98.9 billion in 2021 and are projected to reach USD 137.1 billion by 2026 [38].

For this work, anthocyanin-rich plant material (soft fruits such as bilberry) was used to deliver a sustainable functional food ingredient concept design for T2DM management. However, this concept could also be used to valorize plant bioactives from agricultural/food waste/by-products [39]. Therefore, they could represent an alternative solution to tackling soft fruit waste by bioactive revalorization. The wild bilberries are also naturally high in readily available (free) sugars and much of these were removed during the flour's fortification process, reducing the glycemic load of the fortified food while adding key bioactives with known health benefits. This finding is of particular interest as anthocyanins are being extensively researched as potential natural remedies for diabetes [40]. Moreover, they could also represent a solution for sugars recovery from fruit waste and various agricultural by-products at the same time. Follow up research is necessary to prove the functionality of these fortified flours in human dietary studies.

5. Conclusions

The current work has successfully established the optimum conditions for the fortification of buckwheat, fava bean and hemp flours with additional bioactives (including anthocyanins, phenolic acids and other flavonoids) relevant for the prevention and maintenance of conditions such as type 2 diabetes mellitus. The flours did not retain any of the free sugars in bilberry and additional sugars present in the flours were removed during the fortification process.

This paper discussed food ingredients naturally rich in protein, dietary fiber, micronutrients and bioactive phytochemicals. Being low in free sugars, there is huge potential for these ingredients to be used by the food and drink industry, especially as active functional ingredient for low glycemic food formulations. Additional work would also be necessary for further product development and to test their efficacy in sugar and/or lipid modulation in human dietary intervention studies before their recommendation in any nutritional therapy. The development of specialized food with potential health-promoting benefits using sustainable food sources will ultimately create the demand for the cultivation of these healthy and sustainable crops and deliver innovative ways to utilize potential agricultural waste streams and co-products.

Author Contributions: Conceptualization, M.N. and W.R.R.; methodology, M.N., W.R.R. and J.S.C.; formal analysis, J.S.C., N.J.V. and G.J.D.; data curation, M.N., J.S.C. and G.J.D.; writing—original draft preparation, M.N. and W.R.R.; writing—review and editing, M.N. and W.R.R.; visualization, M.N. and W.R.R.; supervision, M.N. and W.R.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Scottish government’s Rural and Environment Science and Analytical Services Division (RESAS).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge Viv Buchan from the Analytical Department, The Rowett Institute, University of Aberdeen, for her help with the sugar content determinations.

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

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