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Fortification of blackcurrant juice with crowberry: Impact on polyphenol composition, urinary phenolic metabolites, and postprandial glycemic response in healthy subjects

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ABSTRACT

Functional drinks enhanced with putatively bioactive polyphenols are receiving extensive interest due to their potential health benefits. A randomized, controlled, double-blind cross-over study design was used to assess postprandial glycemic responses to a basic blackcurrant juice and a blackcurrant juice fortified with crowberry powder (respective polyphenol contents 159 and 293 mg/100 mL), both sweetened with sucrose. Concurrently, we studied the presence of polyphenol-derived metabolites in plasma and urine to confirm their bioavailability. Urinary metabolites characteristic of cyanidin anthocyanins (such as dihydroxybenzoic acid sulphate and dihydroxyphenylacetic acid sulphate) increased after intake and were present in higher levels after intake of the fortified juice. Compared to the basic juice, the fortified juice elicited slightly attenuated and sustained plasma glucose and insulin responses. In conclusion, fortification of blackcurrant juice with crowberry doubled the polyphenol content and improved postprandial glycemic control in healthy subjects. This gives encouragement for further functional food development.

1. Introduction

Functional drinks containing enhanced levels of putative and proven health-promoting bioactive compounds are receiving extensive interest due to their potential to lower the risk of certain chronic and degenerative diseases, which are accompanied with huge health care costs (McCormick & Stone, 2007; Popkin, 2011). Anthocyanins are one of the most widely distributed class of plant polyphenols and are particularly enriched in berries (Koponen, Happonen, Mattila, & Törrönen, 2007; Wu, Gu, Prior, & McKay, 2004). Along with other polyphenols they continue to receive considerable attention as potential natural and dietary bioactive agents for diverse applications as anthocyanin-rich foods have been demonstrated to provide beneficial protective function against certain cancers (Jing et al., 2008; Thomasset et al., 2009; Wang & Stoner, 2008), cardiovascular diseases (Erlund et al., 2008; Toufektsian et al., 2008), type 2 diabetes (Rayalam, Della-Fera,

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& Baile, 2008; Sasaki et al., 2007; Takikawa, Inoue, Horio, & Tsuda, 2010), obesity (Prior et al., 2008; Rayalam et al., 2008) and age-related macular degeneration (Milbury, Graf, Curran-Celentano, & Blumberg, 2007). A significant increase in plasma anthocyanin concentrations and antioxidant capacities was observed following consumption of an anthocyanin-rich juice (Mertens-Talcott et al., 2008; Pedersen et al., 2000) suggesting that part of the health beneficial roles of anthocyanins may be related to their free-radical scavenging function. However, many of the potential health promoting properties may be independent of antioxidant activity, and anthocyanins may directly or indirectly exert their health benefits by interacting with key signal transduction pathways relevant to disease processes (Joseph et al., 2003; Williams et al., 2008).

However, there are limited *in vivo* and clinical intervention studies available to support the proliferation of *in vitro* studies. In our previous studies, we showed that berries attenuated the postprandial glucose and insulin responses to sucrose in healthy subjects (Törrönen et al., 2010, 2012). This beneficial impact on glucose metabolism is supported by data demonstrating that a bilberry anthocyanin-rich extract ameliorated hyperglycemia and insulin sensitivity in type 2 diabetic mice (Takikawa et al., 2010). Furthermore, supplementation with 300 mg of berry anthocyanins (blackcurrant + bilberry) for 3 weeks in a study comprising 120 subjects exhibited inhibition of NF-kB transactivation and decreased plasma concentrations of pro-inflammatory factors (Karlsen et al., 2007). Short-term supplementation of blueberry anthocyanin extract was also shown to improve memory function in older human population trials (Krikorian et al., 2010).

Clinical studies suggest that at least a proportion of the polyphenols or their metabolites from anthocyanin-rich food/drinks may be sufficiently bioavailable to reach target cells. However, the bioavailability of anthocyanins *per se* is very low with generally less than 0.1–1% of orally administered anthocyanins recovered in the urine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005; McGhie & Walton, 2007). Nevertheless, bioabsorption is also very rapid with maximum plasma anthocyanin concentrations occurring at 15–60 min and excretion broadly complete within 6–8 h.

Food companies are currently focusing efforts for the development of functional drinks which contain significant amounts of distinct types of bioactive compounds (Borges, Degeneve, Mullen, & Crozier, 2010; Gonzáles-Molina, Moreno, & García-Viguera, 2009) as the global functional drinks market is forecast to have a value of $62B in 2015, an increase of 29% since 2010 (Anon, 2011). Blackcurrants (*Ribes nigrum*) are widely grown for juice processing. However, it is reported that their anthocyanins are somewhat sensitive to process degradation (Hollands et al., 2008), and therefore some of the health beneficial properties may be lost during processing. Consequently, introducing additional anthocyanins into blackcurrant products from other sources is one strategy to maintain and/or increase health beneficial properties in blackcurrant juices and other products. As a result of their high and diverse anthocyanin content, wild berries are a very interesting source of incorporating polyphenols into blackcurrant and other berry products. Crowberry (*Empetrum nigrum*) is a particularly interesting wild berry due to its high levels of different anthocyanins (Koskela et al., 2010; Ogawa et al., 2008), an interesting profile of proanthocyanidins (Hellström, Törrönen, & Mattila, 2009) and flavonols (Laaksonen, Sandell, Järvinen, & Kallio, 2011).

In this work, the aim was to study postprandial glucose and insulin responses after consumption of a basic blackcurrant juice and a blackcurrant juice fortified with crowberry powder, both sweetened with sucrose. Concurrently, we studied the presence of polyphenol-derived metabolites in plasma and urine to confirm their bioavailability.

2. Materials and methods

2.1. *Preparation of juices*

The basic blackcurrant juice was made by 1:10 dilution of a juice concentrate (65 Brix, *R. nigrum*). The fortified juice was prepared by adding crowberry (mountain bilberry, *Empetrum nigrum*) powder 100 g/L in the basic juice. The juice concentrate and the berry powder were kindly provided by Kiantama Oy (Suomussalmi, Finland). Sucrose (50 g/L) was added to both juices.

2.2. *Analyses of polyphenols and sugars in juices*

Anthocyanins were analyzed as their intact compounds. A sample of juice (10 mL) was transferred into 50 mL volume flask which was filled with acidified aqueous methanol (65% methanol, 5% formic acid). The diluted sample was filtered through a 45 μm membrane filter into a high-performance liquid chromatograph (HPLC) vial. The analytical system consisted of an Agilent 1100 series HPLC equipped with a diode array detector (DAD). Anthocyanins were separated on a 150 × 4.6 mm i.d., 5 μm, Gemini C18 column with a C18 guard column. The temperature of the column oven was set at 35 °C. The mobile phase consisted of 5% aqueous formic acid (solvent A) and acetonitrile (solvent B) and the flow rate was 1.0 mL/min. Elution was started isocratic at 5% B for 5 min, then a linear gradient to 13% B in 5 min, a linear gradient to 18% B in 10 min and, after that, to 80% in 2 min, isocratic for 3 min and back to the starting point in 2 min. The injection volume was 10 μL. Calibration curves for anthocyanins were generated from authentic standard compounds of cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, and delphinidin-3-rutinoside (Polyphenols Laboratories AS, Sandnes, Norway) at a detection wavelength of 518 nm. However, since other anthocyanins were also observed, it was decided to quantify all anthocyanins against an external standard of cyanidin-3-glucoside to obtain comparable results. Other standard compounds were used mainly to aid identification. Four replicates were analyzed from the samples. Additionally, the samples were analyzed by Thermo Finnigan Surveyor HPLC connected to a Finnigan MAT ion trap mass spectrometry. An ESI interface in positive ionization mode was used under full scan (*m*/*z* 100–800). Anthocyanin concentrations are given as cyanidin-3-glucoside equivalents.

Flavonol glycosides were hydrolyzed by refluxing the freeze-dried samples in 1.2 M HCl in 50% aqueous methanol

for 1 h according to Hertog, Hollman, and Katan (1992). Flavonoid aglycones were identified and quantified using an Agilent 1100 Series HPLC equipped with DAD. The analytical column was Nova Pak C18 (150 × 3.9 mm i.d., 4 μm, Waters, Milford, MA) protected with the manufacturer's precolumn. The mobile phase consisted of 0.05 M phosphate buffer (A) at pH 2.4 and methanol (B) (5–60% B in 50 min followed by 60–90% B in 6 min). The wavelength used for the quantification was 370 nm for isorhamnetin, kaempferol, myricetin and quercetin. For identification purposes, UV/vis spectra were recorded at 190–600 nm.

Phenolic acids were analyzed as aglycones according to a method of Mattila and Kumpulainen (2002) as modified by Mattila, Hellström, and Törrönen (2006). Briefly, phenolic acids were liberated from the parent compounds and matrix by alkali and acid hydrolyses followed by extraction with a mixture of diethyl ether and ethyl acetate. After that the analytes were concentrated by vacuum evaporation, and finally analyzed by HPLC–DAD.

Proanthocyanidins were analyzed from freeze-dried samples according to a thiolytic method described by Hellström and Mattila (2008). Briefly, proanthocyanidins were depolymerised by methanolic hydrochloride in the presence of a strong nucleophile (benzylmercaptan). After that the reaction products were analyzed by HPLC–DAD–FLD. Terminal units of proanthocyanidins were liberated as free flavan-3-ols and the extension units as flavan-3-ol benzylthioethers.

Sugars were analyzed by an Agilent series 1100 HPLC equipped with refractive index detector. Briefly, the sugars were extracted with water and cleaned-up by C18 solid phase extraction cartridge (Waters, Milford, MA) as described by the Nordic Committee on Food Analysis (No. 148/1993). A Luna NH_2 (150 × 3 mm, 5 μm, Phenomenex, Torrance, CA) analytical column was used with acetonitrile–water (75:25) as mobile phase.

2.3. *Human study*

Sixteen healthy volunteers were recruited from the staff of the University of Eastern Finland by an e-mail advertisement. At the screening visit, the health status and medical history of the volunteers were examined by anthropometric measurements, routine blood chemistry and a structured interview. Fourteen subjects (11 women and 3 men) were eligible and completed the study. The Research Ethics Committee of the Hospital District of Northern Savo (Finland) approved the study, and written informed consent was obtained from all subjects.

In this randomized, controlled, double-blind cross-over study each subject participated in two 8-h postprandial tests, on separate days, at least 5 days apart. The basic and fortified blackcurrant juices were served in a randomized order. The day before the test the subjects were instructed to follow a low-polyphenol diet. During the test, a low-polyphenol lunch was served at 4 h and a snack at 6 h. Water (300 mL) was served at 3, 4 and 6 h.

The experiments were started in the morning after a 12-h fast. An intravenous catheter was inserted in the antecubital vein of the arm and the fasting sample was drawn. After consumption of the juice (300 mL), blood samples were collected at 15, 30, 45, 60, 90, 120, 150 and 180 min for measurements of plasma glucose (in citrate–fluoride tubes), insulin and polyphenolic metabolites (in EDTA tubes). Additional samples were collected at 4, 6 and 8 h for polyphenolic metabolites only. The samples were frozen immediately after separation of plasma, and stored at −70 °C until analysis. Baseline 12-h urine was collected during the night before the test, and 0–2, 2–4, 4–6 and 6–8 h samples were collected after consumption of the juice, and the volumes were measured. Aliquots of the samples were frozen at −70 °C and used for analyses of phenolic metabolites. Blood samples could not be collected from one subject, and another subject did not provide urine samples.

Plasma glucose concentrations were analyzed with the hexokinase method using Konelab System reagents and Konelab 20 XTi clinical chemistry analyzer (Thermo Fischer Scientific, Vantaa, Finland). Plasma insulin was determined with an immunoluminometric assay using ADVIA Centaur® Insulin IRI reagents and Siemens ADVIA Centaur® chemiluminescence immunoanalyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).

2.4. *Identification of phenolic compounds in plasma and urine*

Plasma samples were defrosted and proteins precipitated by the addition of acetonitrile containing 0.2% formic acid to a final proportion of 75% v/v. An internal standard of morin (2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) at 10 ng/mL was added. After vortex mixing and incubation on ice for 30 min, the samples were centrifuged (16,500 rpm, 10 min, 4 °C), the supernatants were removed. These were dried in a Speed-vac to remove acetonitrile, then frozen and freeze-dried. The dried samples were resuspended in 100 μL of 5% aqueous acetonitrile containing 0.1% formic acid. This procedure was based on the method outlined by Day et al. (2001) and used by Borges et al. (2010) and achieved recoveries of 78–88% for dosed standards (1 μg/mL cyanidin-3-*O*-glucoside, myricetin-3-*O*-glucoside and chlorogenic acid) and approx. 95% for morin. Samples were analyzed by LC–MS as described below for the urine samples.

Urine samples were defrosted, mixed by vortex and then made up to 10 ng/mL with a stock solution of an internal standard (morin) and prepared in filter vials (Whatman Mini-Unipreps, 0.45 μm) for LC–MS.

Samples (20 μL injections) were analyzed in duplicate on a LCQ–DECA LC–MS system (ThermoFinnigan Ltd.), comprising a Surveyor autosampler, pump and photodiode array (PAD) detector and a Thermo mass spectrometer ion trap. The PAD scanned three discrete channels at 280, 365 and 520 nm. The LCQ–DECA was fitted with electrospray ionization (ESI) interface and was used with full scan (80–2000 *m/z*) in negative mode. The reverse phase separation used a Synergi 4 μm Hydro C18 (150 × 4.6 mm, 4 μm) column (Phenomemex Ltd.) with a linear gradient from 5% B (0.1% formic acid in acetonitrile) in A (0.1% aqueous formic acid) to 40% B over 25 min, then to 100% B at 30 min. The flow rate was 0.4 mL/min.

Selected samples were run under near-identical conditions on an LTQ-Orbitrap mass spectrometer (ThermoFinnigan Ltd.)

with accurate mass scanning to confirm putative peak identities.

Peaks that increased after juice consumption were identified by comparison of the PDA traces using full scan mode and also the different UV channels using the Xcalibur software. As most juice-specific peaks strongly absorbed at 280 nm, they were quantified using their peak area at 280 nm and expressed as peak areas.

2.5. Statistical analyses

The glucose and insulin data were analyzed by using the SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). The incremental areas under the curve (AUCs) were calculated by using GraphPad Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA), ignoring the area below the baseline (0 min) concentration. Data are given as mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated. Linear mixed-effects modelling was used to compare the effects of the juices (time × treatment interaction), and differences at individual time points were tested by post-hoc analysis. Histograms were used for checking normality of model residuals, and logarithmic transformation was used for non-normally distributed data. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Polyphenol composition of the juices

Anthocyanins were the most abundant polyphenol group in the basic and crowberry-fortified blackcurrant juices, fol-

Table 1 – Polyphenols (mg/100 g) in basic and fortified blackcurrant juices.

	Basic blackcurrant juice	Blackcurrant juice fortified with crowberry
Anthocyanins	89.8 ± 0.6 (58%)[a]	178.8 ± 1 (63%)
Cyanidin-3-galactoside	ND	16.0 ± 0.2
Cyanidin-3-glucoside	6.53 ± 0.04	5.94 ± 0.07
Cyanidin-3-rutinoside	34.9 ± 0.3	
Cyanidin-3-rutinoside/cyanidin-3-arabinoside[b]		34.8 ± 0.2
Delphinidin-3-galactoside	ND	18.6 ± 0.2
Delphinidin-3-glucoside	11.3 ± 0.1	8.46 ± 0.07
Delphinidin-3-rutinoside	35.0 ± 0.2	
Delphinidin-3-rutinoside/delphinidin-3-arabinoside[b]		31.1 ± 0.1
Petunidin-3-galactoside	ND	10.9 ± 0.2
Petunidin-3-glucoside	ND	1.16 ± 0.06
Petunidin-3-rutinoside	0.92 ± 0.06	
Petunidin-3-rutinoside/petunidin-3-arabinoside[b]		2.16 ± 0.13
Pelargonidin-3-rutinoside	Traces	Traces
Peonidin-3-galactoside	ND	8.89 ± 0.14
Peonidin-3-rutinoside	0.54 ± 0.01	
Peonidin-3-rutinoside/peonidin-3-arabinoside[b]		2.16 ± 0.13
Malvidin-3-galactoside/peonidin-3-glucoside[b]	ND	29.9 ± 0.2
Malvidin-3-glucoside	ND	0.27 ± 0.08
Malvidin-3-arabinoside	ND	3.43 ± 0.15
Complex anthocyanins (including acylated forms)	0.63 ± 0.03	5.07 ± 0.11
Flavonols	5.25 ± 0.20 (3%)	9.04 ± 0.53 (3%)
Myricetin	3.21 ± 0.16	4.85 ± 0.31
Quercetin	1.63 ± 0.02	3.28 ± 0.07
Kaempferol	0.41 ± 0.02	0.40 ± 0.10
Isorhamnetin	ND	0.51 ± 0.07
Proanthocyanidins (incl. monomeric flavan-3-ols)	50.4 ± 3.1 (33 %)	63.7 ± 4.8 (23 %)
Procyanidin (%)	30 ± 3	38 ± 2
Prodelphinidin (%)	70 ± 3	62 ± 2
Average degree of polymerisation	12.5 ± 0.8	11.3 ± 0.4
A-type linkages (%)	ND	3.3 ± 0.1
Phenolic acids	8.97 ± 0.07 (6%)	29.9 ± 0.9 (11%)
p-Hydroxybenzoic acid	0.53 ± 0.01	0.68 ± 0.06
Protocatechuic acid	1.08 ± 0.02	5.66 ± 0.17
Vanillic acid	0.17 ± 0.01	2.17 ± 0.12
p-Coumaric acid	2.54 ± 0.04	6.73 ± 0.34
Caffeic acid	2.07 ± 0.03	3.22 ± 0.16
Ferulic acid	0.81 ± 0.01	0.82 ± 0.04
Sinapic acid	0.38 ± 0.01	0.45 ± 0.02
Gallic acid	1.39 ± 0.05	3.66 ± 0.27
Syringic acid	ND	6.53 ± 0.11

[a] Percentual proportion of all polyphenols analyzed.
[b] Co-eluting anthocyanins.

lowed by proanthocyanidins, phenolic acids and flavonols (Table 1). The crowberry-fortified juice contained much higher contents of anthocyanins (178.8 mg/100 g) than the basic juice (89.8 mg/100 g). The identification of anthocyanins was based on MS spectra and literature. The anthocyanin profile of blackcurrant juice was dominated by rutinosides of delphinidin ($M^+ = 611$, rt = 11.9 min) and cyanidin ($M^+ = 595$, rt = 12.8 min) followed by the corresponding glucosides ($M^+ = 465$, rt = 11.3 min and $M^+ = 449$, rt = 12.3 min, respectively) which agreed with previous studies on blackcurrant juices (Landbo & Meyer, 2004; Mattila et al., 2011). Rutinosides of petunidin ($M^+ = 625$, rt = 13.6 min), peonidin ($M^+ = 609$, rt = 14.5 min), and pelargonidin ($M^+ = 579$, rt = 13.8 min) were tentatively identified as minor anthocyanins which also agreed with earlier studies on blackcurrant anthocyanins (Borges et al., 2010; Ogawa et al., 2008; Wu et al., 2004). The sharp gradient in the end of HPLC run produced two minor anthocyanin peaks in the chromatogram, which were identified as acylated anthocyanins (Wu et al., 2004), namely cyanidin-3-(6-coumaroyl)-glucoside ($M^+ = 611$, rt = 23.3 min) and petunidin-3(6-coumaroyl)-glucoside ($M^+ = 595$, rt = 22.7 min).

The anthocyanin profile was much more complicated in the blackcurrant juice fortified with crowberry than in the basic blackcurrant juice. The HPLC method could not separate all anthocyanins; for example the rutinosides and arabinosides of the same anthocyanidin co-eluted (Table 1). However, according to MS-based single ion monitoring data, the rutinosides of cyanidin and delphinidin clearly dominated over arabinosides and it could be concluded that these two main anthocyanins of blackcurrant were also the major anthocyanins in the fortified juice. Malvidin-3-galactoside ($M^+ = 493$, rt = 14.1 min) was the next abundant anthocyanin followed by galactosides of delphinidin ($M^+ = 465$, rt = 10.7 min), cyanidin ($M^+ = 449$, rt = 11.7 min), petunidin ($M^+ = 479$, rt = 12.6 min), and peonidin ($M^+ = 463$, rt = 13.4 min). Arabinosides and glucosides of the same anthocyanidins were detected as minor anthocyanins which agreed with previous studies on crowberry anthocyanins (Kellogg et al., 2010; Koskela et al., 2010; Laaksonen et al., 2011; Ogawa et al., 2008).

Myricetin was the main flavonol in both juices, followed by quercetin (Table 1) which agreed with the previous studies on blackcurrant (Koponen et al., 2008) and crowberry (Laaksonen et al., 2011). In the basic blackcurrant juice, the measured content of total flavonols (5.25 ± 0.20 mg/100 g) was in close agreement with the recent study on commercial blackcurrant juices, where the contents varied from 0.6 to 7.6 mg/100 mL among different brands (Mattila et al., 2011). The quercetin content was doubled and the total flavonol content nearly doubled in the fortified juice compared to the basic juice. Small amounts of kaempferol were detected in both juices but isorhamnetin was only found in the fortified juice.

Proanthocyanidins in the basic blackcurrant juice were mostly made up of (epi)gallocatechin subunits with ~70% of proanthocyanidins of prodelphinidin type whilst the remainder were (epi)catechin polymers (procyanidins). They were B-type and their average degree of polymerisation was 12.5 ± 0.8. Fortification of the juice with crowberry increased the content of proanthocyanidins by ~25%. The relative proportion of procyanidins was slightly increased while the average degree of polymerisation was slightly decreased (Table 1).

Table 2 – Sugars (g/100 g) in basic and fortified blackcurrant juices.

	Basic juice	Fortified juice
Fructose	2.3	4.2
Glucose	2.2	4.1
Sucrose	4.9	3.7
Total	9.4	12.0

The fortified juice also contained A-type proanthocyanidins which agrees with previous studies on crowberry proanthocyanidins (Hellström et al., 2009; Kellogg et al., 2010).

Coumaric and caffeic acids were the most abundant phenolic acids in the basic blackcurrant juice followed by gallic and protocatechuic acids (Table 1). These four phenolic acids have previously been shown to dominate in blackcurrants (Mattila et al., 2006, 2011; Russell et al., 2009). The fortified juice had ~3-fold higher level of phenolic acids compared to the basic blackcurrant juice. In the fortified juice, the major phenolic acids were *p*-coumaric acid and syringic acid followed by protocatechuic, gallic, and caffeic acids (Table 1). The occurrence of syringic acid in the fortified juice was expected because this phenolic acid has been reported to dominate in crowberries (Mattila et al., 2006).

3.2. *Sugar composition of the juices*

Fructose and glucose concentrations were almost double and the sucrose concentration 24% lower in the fortified blackcurrant juice compared with the basic juice (Table 2). The lower sucrose content in the fortified juice indicates that some of the added sucrose probably was converted to glucose and fructose either via enzymatic or non-enzymatic means. The total sugar concentrations were 9.4 and 12.0 g/100 g in the basic and fortified juices, respectively.

3.3. *Identification of putative phenolic metabolites in plasma and urine*

3.3.1. *Plasma metabolites*

There were few consistent differences between pre- and post-juice intake in the plasma samples. No anthocyanin or flavonol derivatives were detected. One peak increased in many subjects, but not all, following juice intake with properties [RT 19.8; PDA = 270–280; *m/z* M−H = 178.09; $MS^2 = 134.02$ (loss of CO_2)] consistent with hippuric acid (results not shown).

The limit for detection of anthocyanins and flavonols on the LC–MS system employed was defined at around 1 ng/20 μL maximal injection, equivalent to 50 μg/L for cyanidin-3-O-glucoside (e.g. 0.111 μM or 111 nM). The degree of concentration possible from the entire available 8 mL serum sample as supplied was 32-fold. Therefore, the maximal limit of detection of cyanidin-3-O-glucoside in plasma samples was ~3.5 nM but we could not prepare the samples at this concentration as this did not allow technical replication of samples. As published recoveries for individual anthocyanins are ~2 nM in plasma with rutinosides even lower (e.g. Nakamura et al., 2010), these components were below our limit of detection using the samples available.

3.3.2. *Urinary metabolites*

No anthocyanin-like metabolites could be detected (using absorbance at 520 nm) in the urine samples. This agrees with previous work that indicates that urine levels of anthocyanins or anthocyanidins rarely exceed the nanomolar range (Mullen, Borges, Lean, Roberts, & Crozier, 2010; Walton, Hendriks, Broomfield, & McGhie, 2010). In addition, few peaks that absorbed around 320–360 nm were noted but none were consistently enhanced after juice consumption. Further work with concentrated samples, perhaps using solid phase extraction sample preparation methods (Woodward, Kroon, Cassidy, & Kay, 2009), is required to confirm and quantify the presence of urinary anthocyanin derivatives.

A number of peaks were detected that increased from baseline after juice intake (Fig. 1). These increased components were common to all subjects but their extent of increase varied. Some peaks increased within 0–2 h (e.g. A & B), some increased later (e.g. peak F) and others increased even later (peaks C & D). The same peaks were identified after intake of the basic blackcurrant juice and the blackcurrant juice fortified with crowberry powder.

The majority of increased peaks were putatively identified from their PDA and mass spectral properties (Table 3) as sulphated phenolic metabolites. The presence of such phenolic derivatives in urine after berry intake has been noted previously (e.g. Mullen et al., 2010) and they are characterized by the loss of 80 amu upon fragmentation. Other putative phenolic metabolites, including glucuronides, were identified but these did not increase after juice intake. Hippuric acid (peak F) was identified as a major phenolic derivative and this agrees with the findings of Hollands et al. (2008) who identified this component and its hydroxylated derivatives as major urinary phenolic products after blackcurrant intake. The putative identity of peaks B, C, D, F and G were confirmed using the exact mass facility of the Orbitrap MS (data not shown) with high mass stringency (e.g. delta amu values of ~0.001).

The identification of increased urinary levels of dihydroxybenzoic acid sulphate (peak B) and dihydroxyphenylacetic acid sulphate (peak C) may be characteristic of intake of cyanidin-based anthocyanins (Nurmi et al., 2009). These metabolites result from the B-ring of anthocyanidins after C-ring fission (Vitaglione et al., 2007) which can occur spontaneously at the physiological pH of the small intestine or plasma. However, it is also possible that these metabolites arise after a similar degradation of quercetin-type flavonols also present in the blackcurrant juice, albeit at much lower concentrations than the anthocyanins. However, flavonols are considerably more stable at physiological conditions (Scalbert & Williamson, 2000) and C-ring fission of flavonols would be more likely caused by metabolism by colonic microflora and consequentially evident at a later sampling time. Peak D (putatively identified as catechol sulphate) may also arise from the B-ring of cyanidin-type anthocyanins after fission but could also arise from flavonols or proanthocyanidins.

The increased levels of ferulic and caffeic acid sulphate derivatives (peak E) have been noted previously after anthocyanin intake (Nurmi et al., 2009). Degradation at the C-ring may also produce cinnamic acid derivatives from the A-ring which undergo subsequent methylation and sulphation and this could be the source of the putative dimethoxy cinnamic acid derivative (peak G).

Most of these noted peaks increased within 0–2 h suggesting rapid uptake into the blood, metabolism and excretion of juice-related metabolites in the urine. However, there was considerable inter-individual variation in the pattern and timescales of peak evolution (Supplementary Fig. S1 and S2), which could be influenced by variation in metabolic efficiency. It was interesting that the levels of dihydroxybenzoic acid sulphate (peak B), dihdroxyphenylacetic acid sulphate (peak C) and the catechol sulphate (peak D), that were increased immediately after ingestion (0–2 h), also increased in abundance later (6–8 h). Leaving aside inter-individual variation in the extent of this secondary increase, this later recovery may be caused by a phase of colonic metabolism of juice components, re-absorption of derivatives into the blood, conjugation and excretion in urine.

3.4. *Glucose and insulin responses*

Blood samples for glucose and insulin measurements were available from 13 participants, whose basic characteristics are presented in Table 4.

Plasma glucose and insulin responses were different after consumption of the two blackcurrant juices (Fig. 2). The basic juice caused a rapid rise of glucose with the peak concentration at 30 min, followed by a rapid fall below the baseline level within 2 h. Ingestion of the fortified juice resulted in a slightly lower glucose peak at 30 min and a slow decline during the second hour. However, the overall difference between the juices (time × treatment interaction) was not statistically significant in the mixed model analysis. Comparison at individual time points showed a significant difference only at 90 min ($P = 0.004$). The AUC was larger after ingestion of the fortified juice (85.1 ± 16.4 mmol/L × min; mean ± SEM) compared to the basic juice (64.2 ± 9.1 mmol/L × min), but the difference was not statistically significant.

The insulin responses paralleled the glucose responses (Fig. 2). The overall difference between the juices (time × treatment interaction) was close to statistical significance ($P = 0.062$), and the difference at 90 min was significant ($P = 0.002$). Although the average AUC was larger after ingestion of the fortified juice (9342 ± 1404 pmol/L × min; mean ± SEM) compared to the basic juice (6840 ± 1020 pmol/L × min), the difference was not statistically significant.

4. Discussion

The total contents of the polyphenols analyzed in the basic and fortified blackcurrant juices were 154 and 281 mg/100 g (159 and 293 mg/100 mL), respectively. Anthocyanins were the predominant polyphenols in both juices, followed by proanthocyanidins, phenolic acids and flavonols. Fortification of blackcurrant juice with crowberry doubled the concentrations of anthocyanins and flavonols and tripled the concentrations of phenolic acids. The anthocyanin profile of the fortified juice was much more diverse than that of the basic juice. There was a striking difference between the juices in their content of anthocyanidin galactosides. In the fortified juice,

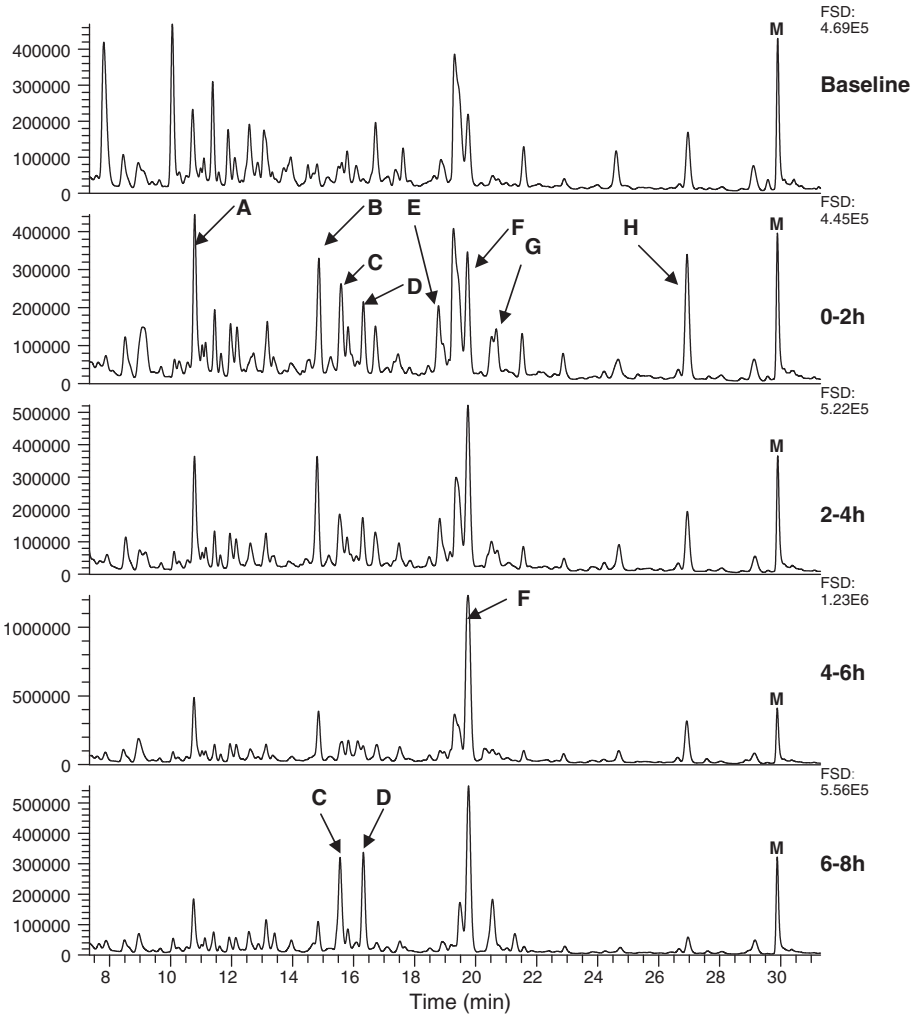

Fig. 1 – Urinary derivatives increasing after fortified blackcurrant juice intake. Arrows denote peaks A–H. M denotes the internal standard, morin. The values in the top right corner of each panel represent the full scale deflection of the PDA detector. The first 7 min of the run contain breakthrough and other peaks common to all urine samples and are omitted for clarity.

Table 3 – Putative identities of urinary peaks after blackcurrant juice intake.

Peak	RT	PDA	[M−H] *m/z*	MS^2	Putative identity
A	10.79	255, 290 (sh)	**244**	**164**, 162, 80	"Phenolic" acid sulphate
B	14.86	255	**233**, 153, 109	189, **153**, 97	Dihydroxy benzoic acid sulphate
C	15.59	265, 295 (sh)	**247**, 167	203, **167**, 123	Dihydroxy phenylacetic acid sulphate
D	16.32	260, 295 (sh)	**189**, 109	**109**	Catechol sulphate
E	18.82	290, 320	**259**, **273 mix**	**179**, **193 resp**.	Mix of ferulic and caffeic acid sulphates
F	19.73	250–280	**178**, 224[a]	**134**	Hippuric acid
G	20.68	260, 290	**287**	**207**, 163	Dimethoxy cinnamic acid sulphate
H	26.90	275	Multiple	–	Unknown

The main MS signals and MS^2 products are in bold. (sh) = shoulder.

[a] The signal at *m/z* 224 may be a formate adduct (+46 amu) of hippuric acid.

47% of the anthocyanins were galactosides, whereas none were detected in the basic juice. Also the concentrations of syringic acid, protocatechuic acid, *p*-coumaric acid, gallic acid, vanillic acid, quercetin and myricetin were increased due to fortification with crowberry.

The serving (300 mL) of the basic and fortified juices provided 277 and 559 mg anthocyanins, 155 and 199 mg proanthocyanidins, 28 and 93 mg phenolic acids, 16 and 28 mg flavonols, and 476 and 879 mg total polyphenols, respectively.

Table 4 – Baseline characteristics of the study participants (mean ± SD, range).

Sex (male/female)	3/10
Age (years)	36 ± 14 (24–61)
Weight (kg)	63.5 ± 13.4 (51–93)
Body mass index (kg/m^2)	22.2 ± 3.0 (18.9–28.7)
Fasting glucose (mmol/L)	5.4 ± 0.4 (4.8–6.2)
Total cholesterol (mmol/L)	4.4 ± 0.7 (2.8–5.5)
LDL cholesterol (mmol/L)	2.4 ± 0.6 (1.4–3.7)
HDL cholesterol (mmol/L)	1.5 ± 0.4 (0.9–2.5)
Triglycerides (mmol/L)	1.1 ± 0.4 (0.5–1.6)

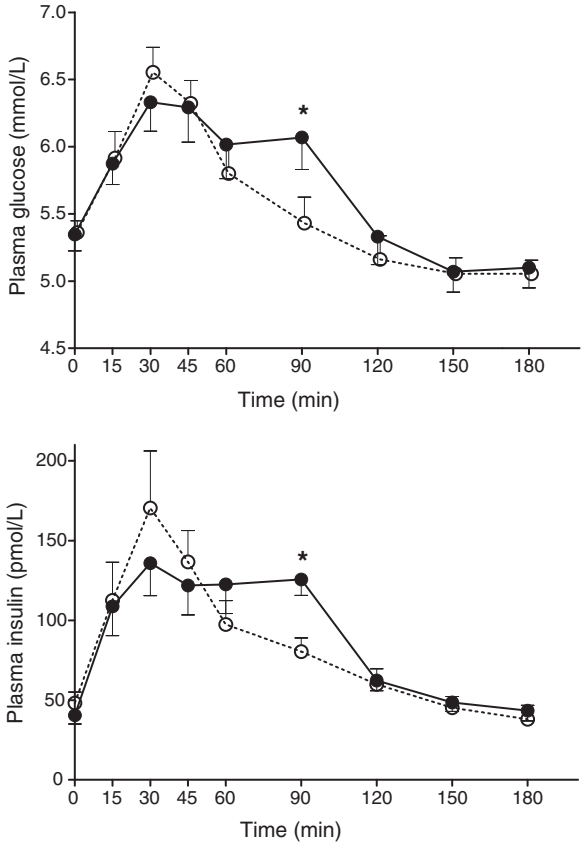

Fig. 2 – Plasma glucose and insulin concentrations (mean ± SEM) after consumption of 300 mL of sucrose-sweetened basic (○) and fortified (●) blackcurrant juices in 13 healthy subjects. *Statistically significant difference between the juices.

Although we were unable to demonstrate consistent changes in plasma polyphenol metabolites, the changes in urinary metabolites suggest bioavailability of polyphenols from the juice. After juice intake, 8 peaks in the LC–MS chromatograms of the urine samples increased rapidly, some within 0–2 h suggesting rapid uptake into the blood, metabolism and excretion of the juice components. No peaks could be identified that were specific for the intake of the fortified over the normal juice. Intake of the fortified juice generally increased the levels of all peaks (A–H) in the urine compared to intake of the basic blackcurrant juice. These increased levels were most apparent for peaks C, D and G but increased levels were noted for all peaks (e.g. at the very least, levels were increased in 8 out of 13 subjects). In many cases, the intake of the fortified juice increased the levels of the peaks but also altered the timescale of their appearance. For example, after intake of the fortified juice, the levels of peak C were clearly increased over 0–2 h compared to the basic juice which may result from the higher intake of cyanidin-type anthocyanins in the fortified juice. The later increase in the levels of peak C, presumably due to colonic metabolism, also occurred in 12 out of 13 subjects but the difference between the basic and the fortified juices was not so obvious (Supplementary Fig. S1). The same pattern was apparent for peaks D and G.

To demonstrate the effects on postprandial glucose and insulin responses, 13 healthy subjects consumed 300 mL of the basic and fortified juices. The juices were sweetened with sucrose (5 g/100 mL), and they also contained the natural sugars of berries, glucose and fructose. According to our analyses, the servings of the basic and fortified juices provided 15 and 12 g sucrose, 14 and 19 g total glucose (either free or from sucrose), and 29 and 38 g total sugars, respectively. The higher amount of glucose in the fortified juice originated from the crowberry powder.

In the intestine, sucrose is hydrolyzed to glucose and fructose by the enzymatic action of α-glucosidase (sucrase). The liberated glucose is absorbed across the intestinal enterocytes via specific transporters. Glucose is mainly responsible for the postprandial rise in plasma glucose and insulin levels, and the contribution of fructose is small (Bantle, 2009). In our study, the AUCs for glucose and insulin responses were 32% and 37% larger, respectively, for the fortified juice than for the basic juice, and this is in a good agreement with the 36% higher intake of total glucose from the fortified juice.

Interestingly, the shapes of the glucose and insulin curves were clearly different after the two juices. The basic blackcurrant juice stimulated rapid rises with peaks at 30 min, followed by a rapid fall to the baseline level (insulin) or even below it (glucose). Consumption of the fortified juice resulted in a different pattern of these responses. Despite the higher intake of sugar, the peaks at 30 min were attenuated. However, due to high between-subject variation the differences were not statistically significant between the juices. During the next hour (30–90 min), glucose and insulin concentrations declined very slowly, resulting in a statistically significant difference between the juices at 90 min. The sustained levels indicate continued absorption of glucose from the fortified juice. At 90 min after ingestion of the basic juice, the plasma glucose concentration had almost returned to the fasting level.

Compared to the basic blackcurrant juice, the fortified juice elicited slightly attenuated but prolonged glucose and insulin responses. The fortified juice had a higher level of anthocyanins and other polyphenols, and the intake of polyphenols was almost double compared to the basic juice. Therefore, it is conceivable that the polyphenols may have reduced or delayed the digestion of sucrose (via inhibition of intestinal α-glucosidase activity) and/or the absorption of glucose. A variety of polyphenols have been shown to inhibit α-glucosidase activity and intestinal glucose transport *in vitro*

(reviewed by Hanhineva et al., 2010). The inhibitory polyphenols include, e.g., anthocyanins, flavonols, proanthocyanidins and phenolic acids. These polyphenols were enriched in the fortified juice. In addition, *in vitro* inhibition of α-glucosidase (McDougall et al., 2005) and intestinal glucose uptake (Manzano & Williamson, 2010) have been reported for extracts of berries (including blackcurrants), and related to their anthocyanin content.

The glucose and insulin responses observed after consumption of the fortified juice may have been influenced by the higher content of fibre originating from the crowberry powder. We did not analyze the fibre content of the juices, but based on the information provided by the manufacturer we estimate that the crowberry powder provided approximately 10 g total fibre/serving, with less than 1 g soluble fibre. It is well established that soluble fibre attenuates postprandial glucose and insulin responses by slowing gastric emptying and retarding glucose absorption due to increased viscosity in the gastrointestinal tract (Dikeman & Fahey, 2006). Since the intake of soluble fibre was small in our study, it is unlikely that these mechanisms would solely explain the responses elicited by the fortified juice.

Meals high in available carbohydrates, such as sucrose, induce early postprandial hyperglycemia, which stimulates insulin secretion resulting in hyperinsulinemia. An exaggerated postprandial insulin response may cause transient late postprandial hypoglycemia, eliciting secretion of counter-regulatory stress hormones (Ludwig, 2002).

Repeated postprandial hyperglycemia and hyperinsulinemia may promote excessive food intake, pancreatic β-cell dysfunction, dyslipidemia, and endothelial dysfunction, and may thereby increase risk for obesity, type 2 diabetes and cardiovascular disease (Ludwig, 2002).

In the present study, consumption of the blackcurrant juice fortified with crowberry resulted in an attenuated and sustained glycemic and insulinemic responses, compared to the basic blackcurrant juice. This improved response was evident in spite of the higher content of available carbohydrate in the fortified juice. We propose that these beneficial properties may be related to the higher polyphenol content in the fortified juice, and explained by reduced digestion of sucrose and/or delayed absorption of glucose from the gut.

As a conclusion, our data show that adding crowberry containing different types of polyphenols into blackcurrant juice doubled the polyphenol content and improved postprandial glycemic control in healthy subjects. On this basis, there are clearly several directions that food and drink producers can follow to enhance product polyphenol content to influence obesity-related metabolic diseases. However, a systematic approach is required to deliver a variety of bioactive phenolic compounds with potentially beneficial glycemic effect into polyphenol-rich beverages without major changes in organoleptic properties (Jaeger, Axten, Wohlers, & Sun-Waterhouse, 2009).

Disclosure statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2012.05.001.

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