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Abbreviated running title: Anthocyanin-rich purple potato extract decreases glycemia

Highlights

- Purple potato extract contained acylated anthocyanins and hydroxycinnamic acids
- The potato extract reduced postprandial blood glucose and insulin peaks and iAUC
- The hypoglycemic effect was seen in 17 healthy men after a high carbohydrate meal
- Acute effects were seen on some of the 90 inflammation markers studied in plasma
- Purple potato phenolics increased FGF-19 levels after a high carbohydrate meal

Abstract

Our recent clinical study suggested that polyphenol-rich purple potatoes lowered postprandial glycemia and insulinemia compared to yellow potatoes. Here, 17 healthy male volunteers consumed yellow potatoes with or without purple potato extract (PPE, extracted with water/ethanol/acetic acid) rich in acylated anthocyanins (152 mg) and other phenolics (140 mg) in a randomized cross-over trial. Ethanol-free PPE decreased the incremental area under the curve for glucose ($p = 0.019$) and insulin ($p = 0.015$) until 120 min after the meal, glucose at 20 min ($p = 0.015$) and 40 min ($p = 0.004$), and insulin at 20 min ($p = 0.003$), 40 min ($p = 0.004$) and 60 min ($p = 0.005$) after the meal. PPE affected some of the studied 90 inflammation markers after meal; for example insulin-like hormone FGF-19 levels were elevated at 240 min ($p=0.001$). These results indicate that PPE alleviates postprandial glycemia and insulinemia, and affects postprandial inflammation.

Keywords: acylated anthocyanins; phenolics; purple-fleshed potatoes; postprandial state; clinical intervention; glycemia; insulinemia; inflammation markers

1 Introduction

High blood glucose level is a risk factor for several metabolic disorders. Especially repetitive, oscillating blood glucose peaks lead to oxidative stress preceding these disorders and are shown to be even more deleterious than high average blood glucose both in healthy and diabetic volunteers (Ceriello et al., 2008). As most of a day is spent in a postprandial state, controlling blood glucose through everyday lifestyle is inevitable for maintaining health. The polyphenolic blue and red colorants of various berries and fruits, the anthocyanins, and various anthocyanin-rich foods, have been suggested to decrease postprandial glucose and/or insulin responses. This has been seen both in healthy (Bell, Lamport, Butler, & Williams, 2017; Castro-Acosta et al., 2016) and diabetic (Hoggard et al., 2013) volunteers after consumption of anthocyanin-rich berries.

Red and purple potatoes provide a rich source of anthocyanins and other polyphenols easy to adopt to an everyday diet. Anthocyanins in potatoes are composed of mainly glycosides of cyanidin and pelargonidin (red varieties) or petunidin, peonidin and malvidin (purple varieties). The glycosides are acylated to phenolic acids, such as *p*-coumaric acid, caffeic acid and ferulic acid. In addition, coloured potatoes are rich in other phenolic compounds, such as chlorogenic acid and hydroxycinnamic acids. (Giusti, Polit, Ayvaz, Tay, & Manrique, 2014; Ieri, Innocenti, Andrenelli, Vecchio, & Mulinacci, 2011)

However, studies on the impact of acylated anthocyanins on postprandial state are still scarce, and the findings have been somewhat controversial. Moser et al., 2018 reported a moderate decrease of blood glucose in healthy subjects after consuming purple potato chips compared to white potato chips, suggesting modulating effects of phenolics of purple potatoes on glycemia. On the other hand, Ramdath et al., 2014 did not find a statistically significant difference in the glycemic response in healthy men after one meal of purple, yellow or white potatoes, but the

glycemic index of the potatoes was seen to be negatively correlated to the polyphenolic content of the potato variety. In animal models, purple potatoes have been documented to lower blood glucose and cholesterol in diabetic rats (Choi, Park, Eom, & Kang, 2013) and to enhance glucose tolerance in obese Zucker rats when compared to white potatoes (Ayoub et al., 2017).

In our recent study (Linderborg et al., 2016) we found that a meal prepared from a purple potato variety (*Solanum tuberosum* L. 'Synkeä Sakari') rich in acylated petunidin and peonidin glycosides lowered postprandial glycemia and insulinemia compared to the control meal prepared from a yellow cultivar (*S. tuberosum* L. 'Van Gogh') in healthy men. In order to remove the effect of different potato varieties on postprandial metabolism in this follow-up study, anthocyanins of Synkeä Sakari were extracted with an aqueous 20 vol-% ethanol solution containing 7 vol-% of acetic acid and purified (Heinonen et al., 2016). A clinical trial was organized to investigate the effect of yellow-fleshed potatoes with and without the addition of the purple potato extract (PPE) rich in acylated anthocyanins on glycemia, insulinemia and inflammation markers in the postprandial state in healthy men. It was hypothesized that PPE lowers the highest blood glucose and insulin peaks and the area under the glucose and insulin concentration curves.

2 Materials and methods

2.1 Clinical nutrition study

2.1.1 Ethics

The study protocol was accepted by the Ethical Committee of the Hospital District of Southwest Finland. The intervention was conducted according to the Declaration of Helsinki, and registered at clinicaltrials.gov as NCT02940080. Each study subject provided their written informed consent.

2.1.2 Study participants

Seventeen healthy men aged between 18 and 45 years from the area of Turku, Finland, participated in the study. At the screening visit, a health interview was conducted, and the body mass index (BMI, 18.5–27 kg/m^2) and blood pressure (<140/80 mmHg) were measured. The volunteers were asked to participate in a fasting-state blood test in the laboratory of the Hospital District of Southwest Finland. The participants were included to the study if the test results were within the following reference values: glucose 4–6 mmol/L, alanine aminotransferase <60 U/L, creatinine <118 µmol/L, thyrotropin 0.4–4.5 mU/L, cholesterol <5.5 mmol/L, triglycerides <2.6 mmol/L and hemoglobin 130–155 g/L. The study participants were non-smokers without regular medication, and they had not participated in other clinical trials or donated blood within two months before the first intervention visit.

2.1.3 Study design

A single-blinded, cross-over study with two potato meals and a wash-out time of at least two weeks was organized. The study participants were asked to refrain from exercise and to consume only foods and drinks low in flavonoids and dietary fiber 48 hours before and 24 hours after the study meal to decrease the effect of baseline diet on their metabolism and digestion. Details on the allowed diet is provided in the Supplementary material (S1). After 12 hours of overnight fasting, the study participants consumed mashed yellow-fleshed potatoes with or without PPE and 300 mL of drinking water as breakfast. Venous blood was collected into lithium-heparin tubes at fasting state, and 20, 40, 60, 90, 120, 180 and 240 minutes after the study meal. Plasma was separated from the blood by centrifugation at 1,500 × g for 15 minutes.

2.1.4 Preparation of the meals

Floury yellow-fleshed potatoes (*Solanum tuberosum* L. 'Afra') were cultivated by Veljekset Kitola Oy, Nousiainen, Finland, and obtained simultaneously from a local grocery store. The purple-fleshed potatoes (*S. tuberosum* L. 'Synkeä Sakari') used for the anthocyanin extraction were cultivated in Kokemäki and Muhos, Finland. The anthocyanins were extracted in the LUT university from 19 kg of purple potatoes using aqueous 20 vol-% ethanol solution containing 7 vol-% of acetic acid and then further purified resulting in 1.2 L of PPE as described by Heinonen et al., 2016.

For the yellow potato portions, the yellow potatoes were washed carefully, cut in half and steam-cooked with peels for 25 minutes (0.7 mL/g of cooking water to fresh weight of potatoes). The cooked potatoes were mashed with a hand-held electric mixer, carefully homogenized and divided into portions. In total, each meal contained 350 g of cooked potatoes with peels and all remaining cooking water (110.9 g). The meals were stored at –18 °C.

In the yellow potato portion, two meal additives were used: 30 mL of PPE (corresponding to extract from 0.48 kg of fresh purple potatoes) was added to produce the study meal, and 30 mL of water was added to prepare the control meal. As PPE originally contained acetic acid (Heinonen et al., 2016) and the sensory properties of the extract needed enhancement, the pH of the two additives was adjusted to 4 by adding 9.1 mmol of acetic acid in the form of synthetic vinegar (Maustaja, Pyhäntä, Finland) to the control meal additive, and by adding 9.5 mmol and 1.7 mmol of food-grade sodium hydroxide (J.T.Baker, Deventer, Holland) to the study meal additive and the control meal additive, respectively. The amount of sodium was standardized between the meals by adding 0.4 g of sodium chloride into the control meal additive. After these additions, the total volume of the study meal and the control meal additives was 40 mL

per meal. The meal additives were stored at –18 °C. Prior to the clinical intervention, a yellow potato portion and a meal additive were taken to a refrigerator to melt overnight. In the morning, the yellow potato portion was heated using a microwave and left to cool down to room temperature. Then, either the study or the control meal additive was added to the yellow potato portion with 10 mL of additional water used to transfer all the residue meal additive from the falcon tube to the meal.

2.2 Blood biomarkers

The plasma glucose and insulin concentrations were analysed in the laboratory of the Hospital District of Southwest Finland as previously described (Linderborg et al., 2016). Using the trapezoidal rule, the incremental areas under the glucose and insulin concentration curves (abbreviated as iAUC) after each meal were calculated until the glucose and insulin levels reached the fasting level. Furthermore, a total of 92 inflammation markers, listed in Table 3, were analysed using cDNA multiplex immunoassay and qPCR giving semi-quantitative results on a log2 scale (the Inflammation panel, Olink Proteomics, Uppsala, Sweden) from the plasma samples collected at the fasting state and 240 min postprandially. Data for two inflammation markers (brain-derived neurotrophic factor and interleukin 1α) were excluded due to technical issues.

2.3 Statistical analyses

Power calculations for required sample size were based on the results obtained in our previous study (Linderborg et al., 2016). Statistical power and effect size were calculated for significant effect of added PPE extract (smaller postprandial plasma glucose in comparison to yellow potato meal; t-test, $p<0.05$) using the G*power software (version 3.1.9). The obtained values

were utilized to calculate the number of volunteers needed for this postprandial test, which turned out to be 15.

Statistical analyses were performed using the IBM SPSS Statistics 23.0 software (SPSS Inc, Chicago, IL) for the glucose and insulin, and RStudio 1.1.456 (RStudio Team, 2016) with Effsize package 0.7.4 (Torchiano, 2018) for the inflammation markers. The significance level was set at 0.05, and the normality of the data was tested using the Shapiro–Wilk test. For normally distributed data, the paired-samples T-test was conducted, and otherwise its non-parametric counterpart, the Wilcoxon signed rank test, was used.

As the inflammation marker data required multiple comparisons, the false discovery rate (type I error) was managed by calculating the effect size measures of Cohen's *d* and *r* score for the parametric and non-parametric tests, respectively. The *r* score was calculated using the equation $r = Z / \sqrt{N}$, in which Z is the test measure of the Wilcoxon signed rank test and N is the total number of observations. The data was interpreted using the following reference values: ≤ 0.2 equals to a small effect size; ≤ 0.5 to a medium effect size, and ≤ 0.8 to a large effect size. The adjusted *p*-values (here, the *q*-values) were calculated using the Benjamini–Hochberg method.

2.4 Characterization of the meals

2.4.1 Materials

For quantification of anthocyanins, flavonol glycosides and hydroxycinnamic acid derivatives, HPLC-grade methanol and formic acid (VWR Chemicals, Radnor, PA) and hydrochloric acid (J.T.Baker, Deventer, Holland) were used. For identification with LC-MS, MS-grade formic acid (Honeywell, Morris Plains, NJ) and acetonitrile (VWR International, Fonteney-sous-Bois,

France) were used. For all analyses, MilliQ-grade water was used, except for the accurate mass analyses in which LC-MS grade water (Merck, Darmstadt, Germany) was used.

2.4.2 Nutrient and starch content of the potato portion

The nutrient and starch content were analysed from the yellow-fleshed potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water) without the meal additives. Starch content was analysed in Eurofins Food Testing Netherlands in Heerenveen using spectrophotometric analyses, and the nutrients (fat, digestible carbohydrates, protein, moisture and ash) and energy were characterized as previously described (Linderborg et al., 2016).

2.4.3 Analysis of ethanol and acetic acid in the purple potato extract

As ethanol and acetic acid were used in the anthocyanin extraction and purification process (Heinonen et al., 2016), their contents in PPE were analysed using gas chromatography. Three replicate samples were taken from PPE and filtrated (0.45 µm, PTFE; VWR, Radnor, PA). The analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Co, Palo Alto, CA), a Hewlett Packard 7673 autosampler and a flame ionization detector. The column was EC-WAX (30 m × 0.53 mm, 1.2 µm, Alltech, Nicholasville, KY). Helium was used as a carrier gas with a total flow rate of 118.0 mL/min in split mode, of which 3.7 mL/min was directed to the column. The injection volume was 0.2 µL. The temperature of the column oven was set at 80 °C, hold for 5 minutes, then increased 10 °C/min until 240 °C and hold for 10 minutes. Quantification was performed using external standard curves prepared from ethanol (Altia Plc, Rajamäki, Finland) and acetic acid (J.T.Baker, Deventer, Holland), respectively.

2.4.4 Analysis of free sugars and organic acids

A representative share of the mashed yellow potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water without the meal additives) was first freeze-dried for 48 hours. Three consecutive samples, 2 g each, of the freeze-dried mashed yellow-fleshed potato portion were extracted using MQ-grade water and then derivatized using Tri-Sil reagent (Pierce, Rockford, IL) as described by Linderborg et al., 2016 in detail.

For the gas chromatographic analyses, a GC-2010 Plus and AOC-20s autosampler (Shimadzu, Kioto, Japan) were used. The samples were injected using AOC-20i autoinjector at 210 °C, and the TMS derivatives were separated with the non-polar poly(dimethyl siloxane) GC column SPB-1 (30 m × 0.25 mm, df 0.25 µm, Supelco, Bellefonte, PA), and detected using a flame ionization detector at 290 °C. The carrier gas was helium (1.90 mL/min). The temperature of the column oven was first 150 °C for 2 min, increased to 210 °C at 4 °C/min, and finally increased at 40 °C/min until 275 °C, which was held for 5 minutes. The peaks of the TMS derivatives were identified using the following external standard compounds: citric acid, malic acid, sucrose (J.T.Baker, Deventer, Holland), ascorbic acid (VWR International, Fontenay-sois-Bois, France), quinic acid (Aldrich, Steinheim, Germany), glucose, and fructose (Merck, Darmstadt, Germany). Quantification was performed by comparing the analyte peak areas with those of the internal standards, which were sorbitol (Sigma–Aldrich, St. Louis, MO) for sugars and tartaric acid (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) for organic acids. Correction factors were obtained by analysing mixtures of the reference compounds and applied in quantification of each compound.

2.4.5 Identification and quantification of anthocyanins

Five consecutive samples of PPE were diluted with MeOH/HCl (99/1, v/v). Anthocyanins of the yellow-fleshed potato portions (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water without the meal additives) were extracted with MeOH/HCl 99/1 four times

from five samples of 1 g of freeze-dried mashed potatoes (Linderborg et al., 2016). The samples were analysed using a high-performance liquid chromatograph LC-10AVP (Shimadzu, Kyoto, Japan) equipped with LC-10AT pumps. 10 µL of a sample was injected with a SIL-10A autosampler and detected at 520 nm with a SPD-M10AVP diode array detector connected with a SCL-M10AVP data handling station. Anthocyanins were separated using a Kinetex Polar C18 column (2.6 µm, 150 × 4.60 mm, Phenomenex, Torrance, CA) at 35°C. The elution solvents consisted of formic acid, acetonitrile and water 5/3/92 (v/v, A) and 5/55/40 (v/v, B), and elution gradient was as follows: 0–5min, 4–20% B; 5–30min, 20–22% B; 30–38min, 22–28% B; 38–42min, 28–32% B; 42–50min, 32–35% B; 50–55min, 35–90% B; 55–58min, 90–35% B; 58–62min, 4% B at flow rate 0.5 ml/min. The anthocyanins were quantified as cyanidin-3-*O*-glucoside equivalents (Extrasynthese, Genay, France) using the external standard method.

For identification, the anthocyanins were first separated with a Waters Acquity Ultra Performance LC system linked to a Waters 2996 DAD detector using the chromatographic method described above, after which the ions were detected with a mass spectrometer (Waters Quattro Premier mass spectrometer with electrospray ionization) operating in the positive ion mode. Full spectra between the mass range of *m/z* 100–1,400 were recorded using the capillary voltage 0.8 kV, the cone voltage 15 V, the extractor voltage 2 V and the RF lens voltage 0.1 V. The ion source temperature was 120 °C, the desolvation temperature 500 °C, the cone gas flow 100 L/h and the desolvation gas flow 650 L/h. Then, the product ions were followed by colliding the selected precursor ions in the second quadrupole at the collision energy of 20 eV and using an argon flow at 0.35 mL/min for further identification purposes. The MS data was handled with the MassLynx 4.1 software (Waters, Milford, MA).

Furthermore, exact masses were measured using the high-resolution Bruker Impact II^{TM} UHR-QqTOF (Ultra-High Resolution Qq-Time-Of-Flight) mass spectrometry in positive

auto-MS/MS mode using electrospray ionization. The compounds were first separated using a Bruker Elute UHPLC equipped with a HPG1300 pump and a diode array detector with the same conditions stated above. The diode array detector response was collected in a range of 190–800 nm. The mass spectrometer parameters were set as follows: the capillary voltage 4.5 kV, the end plate offset 500 V, the nebulizer gas (N_2) pressure 2.0 bar, the drying gas (N_2) flow 8.0 L/min, and the drying gas temperature was 200 °C. The mass range was *m/z* 20 to 1,000. Calibration was carried out by injecting 10 mM sodium formate with 180 µL/min flow rate from a direct infusion syringe pump to the six-port valve for high-accuracy mass experiments in the HPC mode. The mass measurement errors were calculated as the difference between the individually measured accurate mass and the calculated exact mass, given in parts per million. The instrument was controlled and the data was handled with the Compass DataAnalysis software 4.4 (Bruker Daltonik GmbH, Bremen, Germany). In addition, literature was used to aid in the identification (Andersen, Opheim, Aksnes, & Frøystein, 1991; Giusti et al., 2014; Hillebrand, Naumann, Kitzinski, Köhler, & Winterhalter, 2009; Ieri et al., 2011).

2.4.6 Flavonol glycosides and hydroxycinnamic acid derivatives

Flavonol glycosides and hydroxycinnamic acid derivatives were extracted with a modified method (Määttä, Kamal-Eldin, & Törrönen, 2001; Sandell et al., 2009). The samples were prepared in triplicate by first diluting 1 mL of PPE and 1 g of the freeze-dried yellow potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water without the meal additives) into a total volume of 5 mL of MQ water. Then, the samples were extracted using 10 mL of ethyl acetate, mixed vigorously for 1.5 min and centrifuged 1,000 × *g* for 5 min. The ethyl acetate supernatant was collected, and the pellet was extracted three times as described. The ethyl acetate was evaporated using a rotary evaporator at 35 °C. The analytes were diluted in methanol and filtered through 0.45 µm PTFE syringe filters.

The compounds were determined using an HPLC-DAD method described in detail by Linderborg et al., 2016. A wavelength range of 190–600 nm was scanned. Absorption maximum of 320 nm was used for hydroxycinnamic acids and caffeoylquinic acids, and 354 nm was used for flavonols and flavonol glycosides. Caffeoylquinic acid derivatives were calculated as 3-caffeoylquinic acid equivalents, and other hydroxycinnamic acids were calculated as caffeic acid equivalents (Sigma Aldrich, St Louis, MO). Flavonol glycosides were calculated as quercetin-3-*O*-rutinoside equivalents (Extrasynthese, Genay, France).

Flavonol glycosides and hydroxycinnamic acid derivatives were identified by first separating them using a Waters Acquity Ultra Performance LC system linked to a Waters 2996 DAD detector using the chromatographic method described above, and then directing 0.4 mL of the flow to the mass spectrometer (Waters Quattro Premier mass spectrometer with electrospray ionization) operating both in the positive and negative ion modes. The capillary voltage was 3.5 kV (positive) or 3.6 kV (negative), the cone voltage 15 or 22 V, extractor voltage 2 or 4 V, respectively, and RF lens voltage 0.0 V. Source temperature was 120 °C, desolvation temperature 300 °C, cone gas flow 97 L/h and desolvation gas flow 600 L/h. The mass data was collected between the mass range of *m/z* 130–800, and handled with the MassLynx 4.1 software (Waters, Milford, MA).

Identification was confirmed with the high-resolution UHPLC-Q-ToF-MS instrument described in detail in the chapter 2.3.5. The HPLC conditions were as above, and the eluent flow rate from the HPLC to the mass spectrometer was 0.2 mL/min. The flow was ionized using negative electrospray ionization. The capillary voltage was 3.5 kV, the end plate offset 500 V, the nebulizer gas (N_2) pressure 1.4 bar, the drying gas (N_2) flow 9 L/min, the drying gas temperature was 250 °C and collected mass range was *m/z* 20–1,000. The instrument was controlled and the data was processed with the Compass DataAnalysis software 4.4.

3 Results and discussion

3.1 Characterization of the potato portion and meal additives

3.1.1 Composition of the meals

The content of nutrients (Table 1) in the yellow potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water without the meal additives) was similar as in our previous study (Linderborg et al., 2016). The main sugar in the yellow potato portion without the meal additives was glucose (1.4 g) and the main organic acid was citric acid (0.9 g). The study meals contained additional glucose (4.4 mg) and citric acid (9.1 mg) per meal deriving from the supplemented 30 mL of PPE. Both meals contained 0.7 mg of flavonol glycosides and 4.5 mg of hydroxycinnamic acid derivatives from the yellow-fleshed potato portion, and the study meal contained an additional 152.4 mg of anthocyanins and 140.1 mg of hydroxycinnamic acid derivatives from PPE. Furthermore, the study meal contained 0.8 mmol of ethanol and 52.8 mmol of acetic acid derived from PPE.

3.1.2 Identification of anthocyanins

Anthocyanins of 'Synkeä Sakari' were tentatively identified in our previous study (Linderborg et al 2016). For the present study, the chromatographic separation was further improved leading to an increased number of separated anthocyanin peaks (Figure 1A), of which 16 were identified here based on the UV, MS and MS/MS data (Figure 1A, Table 2).

After detecting the molecular ions with mass spectrometry, the product ions from the selected precursor ions were scanned using tandem mass spectrometry. Certain fragmentation patterns were seen. Loss of 162 amu was regarded as a hexose (glucose or galactose), and 454 amu, 470 amu and 484 amu referred to a loss of a rutinose and an acyl group (coumaric acid, caffeic acid and ferulic acid, respectively) from the precursor ions. However, mass spectrometric analyses do not distinguish the structural isomerism without good liquid chromatographic separation

and corresponding reference compounds. Therefore, the coumaric acid was considered to be in the *para* form, the hexose unit was considered to be a glucose, and the glucose was considered to be bonded to the carbon 5 in the A-ring and the rutinose to the carbon 3' in the C-ring as reported in the previous studies utilizing nuclear magnetic resonance spectroscopy for identification of purple potato anthocyanins (Andersen et al., 1991; Hillebrand et al., 2009).

Six anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) were detected. The two major anthocyanins were identified as petunidin-coumaroyl-rutinoside-glucoside and peonidin-coumaroyl-rutinoside-glucoside. Interestingly, the main anthocyanins occurred also in acetylated forms which has not been reported in purple potatoes in literature before. This may have been due to the high concentration of acetic acid in PPE. Furthermore, the peak number 18 remained unidentified due to its low concentration and weak ionization. As its UV spectrum showed a band I absorption maximum at 520 nm, it was tentatively identified and quantified as an anthocyanin.

3.1.3 Identification of flavonol glycosides and hydroxycinnamic acid derivatives

Identification of the detected flavonol glycosides and hydroxycinnamic acid derivatives began by determining the flavonoid class based on the band I absorption maxima in the UV-spectra, and continued with more detailed identification using the retention times, mass spectra, reference compounds when available, and literature. The compounds identified are listed in the Table 2, and the peak numbering refers to the HPLC chromatograms in Figure 1B and 1C. The main hydroxycinnamic acid derivatives in the yellow potato portion and PPE were 3-, 4- and 5-caffeoyl quinic acid isomers (chlorogenic acid, cryptochlorogenic acid and neochlorogenic acid, respectively, $[M-H]^-$ at *m/z* 354) and hydroxycinnamic acids such as caffeic acid and *p*-coumaric acid ($[M-H]^-$ at *m/z* 179 and 163, respectively). From the yellow potato portion, quercetin-3-*O*-rutinoside ($[M-H]^-$ at *m/z* 610), a flavonol glycoside, was found. PPE did not

366 contain flavonol glycosides which may be due to the purification process of PPE after the
367 extraction.

368 PPE contained a caffeoyl quinic acid isomer ($[M-H]^-$ at *m/z* 353), of which the position of the
369 caffeoyl was not defined due to the lack of a reference compound. Furthermore, two isomers
370 of coumaroyl-rhamnosyl-hexoside ($[M-H]^-$ at *m/z* 472) and a coumaroyl-rhamnosyl-acetyl-
371 hexoside ($[M-H]^-$ at *m/z* 514) were identified with the aid of mass fragmentation and tandem
372 mass spectrometry. The structural isomerism of the two coumaroyl-rhamnosyl-hexosides may
373 be in the position of the hydroxyl group of the coumaric acid, and the hexose may be a glucose
374 or a galactose. As coumaroyl-rhamnosyl-hexosides have not been earlier detected in potatoes,
375 they may be breakdown-products of the acylated anthocyanins. Furthermore, two
376 hydroxycinnamic acid amides were found (King & Calhoun, 2005). Feruloyloctodopamine
377 ($[M-H]^-$ at *m/z* 329) was identified both from PPE and the yellow potato portion, and
378 feruloyltyramine ($[M-H]^-$ at *m/z* 313) was found only from the yellow potato portion.
379

380 **3.2 Glycemia and insulinemia**

381 Figure 2 presents the concentrations of plasma glucose (Figure 2A) and insulin (Figure 2B) at
382 the fasting and the postprandial states until 240 minutes after the study meal and the control
383 meal. The incremental area under the glucose curve until the time point of 120 minutes was
384 significantly lower compared to that of the control meal ($p=0.019$). Additionally, the study
385 meal caused a statistically significantly lower glucose response at 20 min and 40 min after the
386 meal compared with the control meal ($p=0.015$ and 0.004, respectively). At 240 min, the
387 glucose response was higher than the response at the corresponding time point after the control
388 meal ($p=0.023$). The iAUC120 min of insulin was significantly lower ($p=0.015$) after the study
389 meal (Figure 2, Supplementary material S2). The study meal caused lower plasma insulin

responses at 20, 40 and 60 minutes after the meal (p=0.003, 0.004, 0.005, respectively), and increased it at 180 and 240 minutes (p=0.004 and 0.006, respectively).

Overall, the study meal modified the postprandial glycemic and insulinemic responses after the meal compared to the control meal by ameliorating the steep increase in the levels of both plasma glucose and insulin at 20–60 minutes. Thereafter, the decrease of both plasma glucose and insulin were slowed down by the study meal.

Several possible pathways may have been involved in the biochemical mechanisms underlying the glycemia modifying effects. Polyphenol-rich extracts from both purple and red cultivars have been shown *in vitro* to decrease the activity of α-glucosidase, which breaks starch down into glucose and maltose during digestion (Ramdath et al., 2014). Moser et al., 2018 reported that purple potato polyphenols inhibit glucose transportation to Caco-2 intestine model cells *in vitro*. In the comprehensive reviews by Hanhineva et al., 2010 and Williamson, 2013, it is stated that polyphenols may modulate intracellular signaling pathways and gene expression related to carbohydrate metabolism. Furthermore, anthocyanin metabolites and degradation products resulting from gut microbiota metabolism may contribute to the health effects of these compounds.

Acetic acid was used to lower the pH of the extraction medium in order to stabilize the potato anthocyanins (Heinonen et al., 2016). The study meal additive contained 52.8 mmol of acetic acid and to adjust the pH to the same value between the study meal and control meal additives, 9.5 mmol of sodium hydroxide was added to the study meal additive, and 9.1 mmol of acetic acid and 1.7 mmol of sodium hydroxide were added to the control meal additive. Amount of sodium was adjusted between the meals by adding 0.4 g of sodium chloride to the control meal additive. Even though the pH of the meal additives were the same, the study meals contained more acetic acid than the control meal due to the high content of acetic acid in PPE caused by

buffering effect of PPE. One dose of vinegar has been shown to lower postprandial glycemia and insulinemia in healthy subjects in a dose-dependent manner (18, 23 and 28 mmol of acetic acid) (Östman, Granfeldt, Persson, & Björck, 2005). Therefore, acetic acid may have partially contributed to the postprandial effects seen in this study.

Despite the indication of hypoglycemic effect of acetic acid, the mechanism involved is not clear. The effect may be connected to the inhibition of α-amylase, enhanced glucose uptake and transcription factors as recently reviewed by Santos, de Moraes, da Silva, Prestes, & Schoenfeld, 2019. Possibly low pH affects the enzyme activities resulting in reduced glycemic response. It is worth to notice that the study designs between our study and the cited research were different: in the cited research pH values were not adjusted between the meals, whereas in our current study the pH of the meals was carefully adjusted to the same value to minimize the potential effect of different pH on enzyme activities. Furthermore, in our previous study (Linderborg et al., 2016), where no acetic acid was used, a meal of purple potatoes of the same variety showed beneficial effects on postprandial glycemia and insulinemia compared to a yellow potato meal. Finally, this type of anthocyanin-rich purple potato extract could not have been prepared without acidic conditions as anthocyanins are not stable in neutral solutions. Acetic acid was chosen as it is a soft acid generally accepted and used in a variety of food products.

In addition, PPE contained high levels of chlorogenic acid which may also have a glycemic index lowering effect (Bassoli et al., 2008). Consequently, our results may be affected not only by the potato anthocyanins, but also by the difference in the contents of acetic acid and hydroxycinnamic acid derivatives between the study and control meals.

3.3 Inflammation markers

Inflammation marker levels were compared between the two meal types (the study and the control meals) at 240 minutes, and also between the fasting state and the 240 min time point within both meal types. The fasting levels did not differ between the study and the control meals (Table 3). Between the meal types at 240 minutes, the levels of C-C motif chemokine 20 (CCL20, $p < 0.001$) and fibroblast growth factor 19 (FGF-19, $p < 0.001$) were increased by the study meal with a statistically significant difference with large effect sizes. Other markers, which were also increased statistically significantly, but with only small or medium effect size, were eukaryotic translation initiation factor (4E-BP1, $p = 0.045$), C-C motif chemokine ligand 25 (CCL25, $p = 0.045$), interleukine 8 (IL-8, $p = 0.011$), oncostatin-M (OSM, $p = 0.005$) and transforming growth factor alpha (TGF-alpha, $p = 0.045$) after the study meal compared to the control meal at 240 minutes.

Furthermore, the levels of Fms-related tyrosine kinase (Fit3L, $p < 0.001$ and $p = 0.003$), monocyte chemotactic protein 1 (MCP-1, $p < 0.001$ and $p = 0.004$), matrix metalloproteinase 10 (MMP-10, $p < 0.001$ and $p = 0.031$), TNF receptor superfamily member 9 (TNFRSF9, $p < 0.001$ and $p = 0.013$) and TNF-related activation-induced cytokine (TRANCE, $p < 0.001$ and $p < 0.001$) were decreased at 240 min after control meal and study meal, respectively, compared with the fasting state and at 240 minutes. FIt3L, MMP-10, MCP-1 and TRANCE had a large effect size for both meals, and MMP-10 and TNFRSF9 had large effect sizes only in the case of the control meal. The level of interleukin-6 (IL-6), however, was increased at 240 minutes compared with the fasting state, both after the control meal ($p < 0.001$) and the study meal ($p < 0.001$). However, the increase had a large size effect only in the case of the study meal.

Several markers were reduced only after the control meal at 240 min compared with the fasting state. Those with large effect sizes were C-C motif chemokine 20 (CCL20, $p = 0.002$), T cell surface glycoprotein CD5 (CD5, $p = 0.001$), T cell surface glycoprotein CD6 isoform (CD6, $p = 0.001$), C-X-C motif chemokine 10 (CXCL10, $p < 0.001$), interleukin-7 (IL-7, $p = 0.004$), interleukin-10 receptor subunit beta (IL-10RB, $p = 0.003$), urokinase-type plasminogen activator (uPA, $p = 0.001$) and vascular endothelial growth factor A (VEGF-A, $p < 0.001$).

Interestingly, the proinflammatory cytokine IL-6 increased after both meals, as was previously seen after a carbohydrate-rich meal in healthy volunteers (Steinberg, Stentz, & Shankar, 2018). The study meal caused a smaller increase in IL-6 compared to the control meal; however, the difference was not statistically significant between the meals. Furthermore, FGF-19 increased slightly after the study meal without statistical significance but decreased statistically significantly after the control meal. The FGF-19 levels were statistically different between the two meals at 240 min postprandially. FGF-19 is an insulin-like ileum-derived postprandial enterokine regulating bile acid homeostasis (Inagaki et al., 2005) reported to possess anti-diabetic properties as it decreases glucose levels in rodents independently from insulin possibly by converting glucose to lactate (Morton et al., 2013). FGF-19 also increases metabolic rate in high-fat fed mice (Fu et al., 2004), regulates hepatic glucose homeostasis by suppressing gluconeogenesis (Potthoff et al., 2011) and induces glycogen synthesis (Kir et al., 2011). Hence, FGF-19 has been suggested to ameliorate obesity, type 1 and 2 diabetes, bile acid overproduction and hepatocellular carcinoma as recently reviewed (Somm & Jornayvaz, 2018).

Recent studies display evidence of potato phenolics acting as anti-inflammatory agents. Kaspar et al., 2011 studied blood plasma inflammatory marker levels of 12 healthy men before and after a six-week daily consumption of 150 g of white, yellow and purple potatoes. They reported a reduction in IL-6 and CRP levels in men who consumed purple potatoes compared to those consuming white potatoes. Also Zhang et al., 2017 reported a decrease in the

production of IL-8 *in vitro* by adding purple potato extract rich in petunidin-3-*O*-*p*-coumaroylrutinoside-5-*O*-glucoside into TNF-alpha induced Caco-2 cells. The biochemical mechanisms may involve suppression of the NF-κB pathway as activation of the NF-κB leads to elevated levels of pro-inflammatory cytokines and inflammation mediators (Karlsen et al., 2007). Furthermore, the phenolic metabolites and degradation products may have a role in the modulation of inflammation. For example, phenolic metabolites of cyanidin-3-*O*-glucoside were seen to reduce IL-6 levels in an *in vitro* cultivation of human vascular endothelial cells, but the parent compound itself had no effect (Amin et al., 2015). In the current study, the function and biological significance, in relation to nutrition, of postprandial levels of most of the inflammatory mediators investigated are unclear, promoting the need for future studies to reveal the biological relevance of these results. Furthermore, more studies are needed to examine the postprandial behavior of the 90 inflammation markers as it has been scarcely studied so far.

We studied here the postprandial inflammation response in healthy men as acute effects of nutrition on postprandial inflammation response have profound relevance to human health as reviewed by Muñoz & Costa, 2013. Meals have been found to cause acute postprandial inflammation response even in healthy study subjects as high consumption of glucose and fatty acids leads to oxidative stress inducing NFκB mediated inflammation markers. Gregersen, Samocha-Bonet, Heilbronn, & Campbell, 2012 reported that an acute high-carbohydrate meal excessive in calories enhances levels of IL-6 and decreases plasma total antioxidative status and muscle Cu/Zn-superoxide dismutase. It was also discussed that one high-carbohydrate meal may cause more severe inflammatory response than a high-fat meal. Connection of dietary glucose and inflammatory response is also dose-dependent; Dickinson, Hancock, Petocz, Ceriello, & Brand-Miller, 2008 reported that higher glycemic index induce higher inflammatory response. Therefore, thorough investigation of postprandial inflammation status

after one meal in healthy study participants is essential for understanding the health effects of the foods in question.

The statistical differences in the inflammation marker levels between the study and the control meals were moderate. A single meal may not be enough to produce a large impact on the inflammation status of healthy study subjects as seen in our recent publication (Nuora et al., 2018), even though the meals used in our current study were rich in carbohydrates and energy. Secondly, the selected time point 240 min may not have been optimal for measuring all the 90 selected inflammatory markers and it may have been too late for detecting the peak concentration of some inflammation markers. For example, IL-6 and FGF-19 are reported to peak already at 180 minutes (Steinberg et al., 2018) and 160 minutes (Morton, Kaiyala, Foster-Schubert, Cummings, & Schwartz, 2014), respectively, after a high-carbohydrate meal. However, we succeeded in our objective to screen a wide array of inflammation markers, but for better understanding of the postprandial behavior of inflammation mediators, more sampling points would have been beneficial. Lastly, one dose of PPE may have been insufficient for distinguishing more significant acute effects.

4 Conclusions

In this study, we carried out a postprandial cross-over clinical study in which 17 healthy study participants consumed a meal of yellow potatoes with or without the purple potato extract (PPE, extracted with water/ethanol/acetic acid) rich in acylated anthocyanins and hydroxycinnamic acid derivatives. The aim was to investigate whether the ethanol-free purple potato extract affects glycemic, insulinemic and inflammatory responses in healthy human subjects. Our results show that the purple potato extract added to a yellow potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water) suppressed the postprandial plasma glucose and insulin peaks and delayed the decrease in the plasma glucose and insulin levels

thereafter, compared to a meal of yellow potatoes. Blood glucose and insulin did not decrease below the fasting levels in four hours after the study meal as they did after the control meal. Therefore, our study hypothesis was supported. Besides glycemia and insulinemia, we investigated the changes in the postprandial low-inflammation state by screening 90 inflammation markers from the plasma samples of the healthy study subjects at fasting state and at 240 minutes after the meals. The energy- and carbohydrate-rich yellow potato portion with or without PPE showed an inter-treatment effect on inflammation markers, such as the insulin-like hormone FGF-19. As we studied here the acute effects of one meal, long-term effects of purple potato phenolics should be investigated in the future.

In our recent study (Linderborg et al., 2016), we compared the impact of a meal of purple-fleshed potatoes with that of yellow-fleshed potatoes on glycemia and insulinemia; the results suggested that purple potatoes are more beneficial to human postprandial glucose metabolism compared to yellow potatoes. The present study showed the findings are true also of the extract of purple potatoes. Furthermore, our study confirmed extracted potato-derived acylated anthocyanins and other phenolic compounds can be used as bioactive components for improving the postprandial glycemic response after a high carbohydrate meal. To the best of our knowledge, this is the first time such results are reported for a purple potato extract rich in acylated anthocyanins and other phenolics.

In order to study the metabolic impact of the purple potato anthocyanins, we successfully removed the possible effects of different potato varieties on biomarkers by extracting the anthocyanins from the potatoes and adding them into a yellow potato portion which was also used as the control meal. This excluded the effects of for example differences in the content and structure of starch as well as the content of the vitamin C. Study participants acted as their own control in a cross-over manner which decreased the interindividual variation related to parallel studies. The baseline diet was strictly restricted concerning dietary fiber, flavonoids,

dietary supplements and alcohol for two days before the intervention, and one day after the intervention to decrease the effect of baseline on the responses. We screened 90 inflammation markers, of which a majority has not been previously reported in nutrition studies related to potato phenolics. Our study is the first one to demonstrate the upregulation of the postprandial level of FGF-19 after a high-carbohydrate meal by dietary anthocyanins. This is also the first study in which the acute postprandial levels of 90 inflammation markers are studied after a high carbohydrate meal with and without phenolic compounds extracted from purple potatoes. However, our results may be partially affected by the difference in amount of acetic acid, used in the extraction of PPE, between the control and study meal.

As a conclusion, this study shows evidence that the purple potato extract rich in acylated anthocyanins decreases the postprandial glucose and insulin peaks and slows down the decrease of glucose and insulin thereafter. As most of the day is spent in the postprandial state and repetitive, fluctuating high blood glucose peaks are associated with oxidative stress and type 2 diabetes, these findings indicate that increasing the intake of acylated anthocyanins and other phenolics derived from purple potatoes as a part of a versatile and nutritious diet may contribute positively to health. These health-promoting compounds may be cost-effectively received from consuming purple-fleshed potatoes or similar food-grade purple potato extracts used in this study. Purple potato extracts may be produced from the food industry side streams, such as potato peels, and be used as a part of health-promoting functional foods.

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Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1. The nutrient composition (*n*=2), sugars and acids (*n*=3), anthocyanins (*n*=5), flavonol glycosides and hydroxycinnamic acid derivatives (*n*=3) of the yellow-fleshed potato portion (350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water) without the meal additives, and PPE (30 mL, the amount added to the study meal). Values are given as mean ± standard deviation.

Table 2. Identification and quantification of anthocyanins, flavonol glycosides and hydroxycinnamic acid derivatives in the purple potato extract (PPE) and the yellow-fleshed potato portion (YP, 350g of cooked yellow-fleshed potatoes and 110.9 g of cooking water without the meal additives) based on the UV, MS, MS/MS, and Q-ToF-MS data. Shown positive ions are M^+ for anthocyanins and $[M+H]^+$ for hydroxycinnamic acid derivatives. Amounts are given as mg per meal (referring to the anthocyanin-rich purple potato extract, PPE, and the yellow potato portion) ± standard deviation.

Table 3. Average plasma inflammation marker levels at the fasting state and 240 min after the study and control meals analysed using the cDNA-based proximity extension multiplex immunoassay and qPCR. The values are means (n = 17) using an arbitrary, semi-quantitative log2 scale, and variation is given as standard deviation (SD). Differences between the two meals within a time point and between the two time points within each meal were statistically compared using significance level of 0.05 for between-group comparisons. Furthermore, the Benjamini–Hochberg corrected *p*-values (*q*-values) and effect size (Cohen's *d* or *r*, depending on normality of the data), abbreviated here as ES, are listed.

Figures.

Figure 1. HPLC chromatogram A) at 520 nm of purple potato extract (PPE) derived from *Solanum tuberosum* L. 'Synkeä Sakari'; B) at 320 nm of the yellow potato portion *S. tuberosum* L. 'Afra'; C) at 320 nm of the purple potato extract (PPE) from *S. tuberosum* L. 'Synkeä Sakari'. Numbering of the peaks refer to Table 2.

Figure 2. Plasma glucose (A) and insulin (B) concentration (n = 17) after the study meal (■) and the control meal (▲). Values are presented as mean ± standard deviation.