A single supplement of a standardised bilberry (*Vaccinium myrtillus* L.) extract (36 % wet weight anthocyanins) modifies glycaemic response in individuals with type 2 diabetes controlled by diet and lifestyle

(單一補充標準化越橘 (Vaccinium myrtillus L.) 提取物（36% 濕重花青素）可改善受飲食和生活方式控制的 2 型糖尿病患者的血糖反應)

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Abstract

Dietary strategies for alleviating health complications, such as premature vascular disease, associated with type 2 diabetes (T2D) and obesity are actively being pursued as alternatives to pharmaceutical interventions[, 1]. The genus *Vaccinium* (for example, blueberry, bilberry, cranberry) has been used traditionally as a source of folk remedies for established diabetic symptoms.

Berries from this genus are rich in anthocyanins, polyphenols recognised for their ability to provide and activate cellular antioxidant protection and inhibit inflammatory gene expression[, 2 - 4], activities that may contribute to the efficacy of the *Vaccinium* genus as ameliorators for T2D. Consumption of a freeze-dried blueberry beverage for an 8-week period, for example, decreased plasma concentrations of the cardiovascular risk factors oxidised LDL, malondialdehyde and hydroxynonenal. In another trial, bioactives from blueberries improved insulin sensitivity in obese insulin-resistant men and women[, 5 , 6]. In both these studies the investigators reported no change in inflammatory markers following supplementation although

bilberry juice was shown to modulate plasma markers of inflammation C-reactive protein and IL-6 in subjects with increased risk of CVD[, 7]. These beneficial responses from human studies are supported by data that demonstrate long-term beneficial effects of anthocyanins from mouse models of obesity and diabetes[, 8 – 12].

There are also a number of studies *in vitro* and *in vivo* that suggest that polyphenols influence carbohydrate digestion and absorption, resulting in improved postprandial glycaemia in the short term. Polyphenols inhibit intestinal α-glucosidase activity[, 13 – 17] and glucose transport[, 13 , 15 , 18 – 21] *in vitro*. In association with this, polyphenols administered to rodents suppress the elevation of blood glucose concentration after oral administration of mono- and di-saccharides[, 13 – 17 , 19 , 22]. In human subjects, several studies have examined the effect of polyphenols on the postprandial glycaemic response[, 23]. In one study, a test meal of mixed berry purée with sucrose showed a lower plasma glucose concentration after 15–30 min compared with a control matched for sugars[, 24].

Overall, evidence suggests that consuming edible berries, particularly from the genus *Vaccinium*, that have high concentrations of anthocyanins could provide a supplementary intervention to improve glycaemia in subjects with T2D or impaired glucose tolerance. The object of the present study was to investigate whether a single supplementation with a standardised (36 % (w/w) anthocyanins) concentrated bilberry extract could alter glucose metabolism in overweight/obese volunteers with impaired glucose intolerance or T2D compared with a control capsule matched for sugars and to explore the possible mechanisms of action.

Methods

Subjects

Male volunteer subjects (*n* 8; BMI 30 (SD 4) kg/m^2; aged 62 (SD 5) years) with T2D controlled by diet and lifestyle alone or with impaired glucose tolerance (Table 1) were recruited from the Aberdeen area of the UK. Subjects were only included if they were not on any special religious or prescribed diet and had a stable weight. Medical exclusion criteria included chronic illnesses, such as thromboembolic or coagulation problems, thyroid disease, renal or hepatic disease, severe gastrointestinal disorders, pulmonary disease (for example, chronic bronchitis, chronic obstructive pulmonary disease), alcohol or any other substance abuse, eating disorders or psychiatric disorders. Volunteers were also excluded if they were taking oral steroids, tricyclic antidepressants, neuroleptics, anticoagulants, digoxin and anti-arrhythmics, or chronically using anti-inflammatories (for example, high doses of aspirin, ibuprofen) or nutrient supplements. These criteria were checked with each participant's primary care physician. All subjects provided informed written consent before inclusion in the study, which was approved by the North of Scotland Research Ethics Committee (NOSREC). The study was registered at clinicaltrials.gov no. NCT01245270 and was conducted according to the guidelines laid down in the Declaration of Helsinki. On both visits, all anthropometric measurements were made following an overnight fast.

Table 1.

Baseline characteristics of the lean and overweight diabetic study volunteers (*n* 8)

(Mean values and standard deviations)

	Mean	SD
Age (years)	62·13	4·55
BMI (kg/m^2)	30·32	4·38
Height (cm)	174·35	7·37
Body weight (kg)	92·33	15·05
Body weight:height ratio	0·53	0·08
Waist circumference (cm)	105·9	11·44
Hip circumference (cm)	105·1	5·83
Waist:hip circumference ratio	1·01	0·06
Blood pressure (mmHg)		

	Mean	SD
Systolic	142·5	15·70
Diastolic	81·1	7·47
Plasma cholesterol (mmol/l)	4·29	0·91
Plasma glucose (mmol/l)	7·16	1·41
Plasma HDL-cholesterol (mmol/l)	1·18	0·39
Plasma LDL-cholesterol (mmol/l)	2·59	0·87
TAG (pmol/l)	1·42	0·51
NEFA (mmol/l)	0·59	0·14
Fasting plasma insulin (pg/ml)	407·70	208·41
HOMA-IR	3·35	2·19

	Mean	SD
HOMA-β	405	190

HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function.

Study design

In a cross-over design, volunteers (*n* 8) were randomised and double-blinded into two groups matched for BMI as well as age and given a single capsule of either 0·47 g of Mirtoselect® (a standardised bilberry extract (36 % (w/w) anthocyanins)) which equates to about 50 g of fresh bilberries formulated in gelatin capsules or a control capsule consisting of microcrystalline cellulose in an opaque gelatin capsule, followed by oral glucose tolerance testing (OGTT). The reverse procedure was conducted following a 2-week washout period. The volunteers were asked to consume a low-phytochemical diet 3 d before taking the capsule and for the 24 h after taking the capsule on both occasions. In addition the volunteers were asked to record what they ate over the same period in a food diary to ensure that they adhered to the low-phytochemical diet. Subjects were reimbursed travelling expenses on completion of the study.

Oral glucose tolerance testing

In the evening before the test on each visit the volunteers were asked to consume the same low-phytochemical meal. Volunteers were fasted (10–12 h) overnight before the OGTT. Venous blood samples were taken through an indwelling cannula inserted into a forearm vein at –15, –10 and –5 (fasted) and at 15, 30, 45, 60, 90, 120, 150 and 300 min after consuming 75 g of Polycal liquid (carbohydrate, 61·9 %; polysaccharide, 49·2 %; sugars, 12·2 %; glucose, 0·6 %; maltose, 11·6 %; http://www.nutricia.co.uk). Polycal was selected as the main carbohydrate as it is in the form of polysaccharides and this is closer to normal dietary consumption than glucose only. It is often used in hospitals as a standard glucose load for OGTT as it is more palatable. The volunteers consumed the appropriate capsule (0 min), glucose load and a further sample of water (70 ml) within 3 min. For those volunteers taking the control capsule, additional sugar (fructose and dextrose/glucose) was added double-blinded to the water to match the free sugar content of the Mirtoselect® capsules. Movement during the 300 min OGTT was kept to a minimum. Plasma glucose concentrations were measured in triplicate using an automated clinical analyser (Kone Oyj). Plasma insulin was measured in duplicate using an ELISA assay (Mercodia). The inter- and intra-assay CV were 2·6–3·6 and 2·8–3·4 %, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was measured using the fasting glucose and insulin values.

Metabolic profile

An automated clinical analyser (Kone Oyj) was used for the analysis of plasma glucose, TAG, LDL, HDL, NEFA and cholesterol using commercial kits (Microgenics GmbH).

Mirtoselect®

Mirtoselect® is a standardised hydro-alcoholic extract of *Vaccinium myrtillus* L. berries (Mirtoselect®; Indena S.p.A.) containing 36 % (w/w) of anthocyanins by HPLC. The extract is obtained from frozen ripe fruit of *V. myrtillus* L. harvested when ripe during July to September. Predominant anthocyanin constituents are delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, cyanidin-3-galactoside and cyanidin-3-glucoside (Indena datasheet). Other anthocyanins include cyanidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside and malvidin-3-arabinoside. Mirtoselect® also contains other polyphenols (phenolic acids, flavonols, proanthocyanidins; about 18 %), carbohydrates and aliphatic organic alcohols (about 29 %), fats (about 0·04 %), N compounds (about 1 %), ash (about 0·7 %), with the remaining 15 % undefined. The main sugar composition of the Mirtoselect® is fructose, 13 %; glucose, 14 %; and sucrose, 4 % (w/w). No side effects of the Mirtoselect® supplementation were noted. Mirtoselect® batch no. 29993/M2 was used in the present study.

Plasma ELISA

Plasma was collected in EDTA tubes containing aprotinin with dipeptidyl peptidase-4 (DPP-IV) inhibitor (Millipore Ltd) added at 10 µl/ml and stored at –70°C until analysis. Plasma glucagon, amylin, monocyte chemotactic protein-1 (MCP-1), gastric inhibitory polypeptide (GIP) and insulin were detected using the commercial Milliplex multiplex ELISA kit (HMHMAG-34K) according to the manufacturer's instructions (Millipore Ltd). All samples were run in duplicate. The minimum level of detection of glucagon was 6 pg/ml and the intra- and inter-assay CV were 3 and 7 %, respectively. The recovery of glucagon added to plasma was approximately 98 %. Similar values for all the other analytes can be found on the Millipore website (www.millipore.com/).

Glucagon-like peptide-1 RIA

Glucagon-like peptide-1 (GLP-1) concentrations in plasma were measured by RIA after extraction of plasma with 70 % ethanol (v/v, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390 which has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 7–36 amide and cross-reacts less than 0·01 % with carboxy-terminally truncated fragments and 89 % with GLP-1 9–36 amide, the primary metabolite of DPP-IV-mediated degradation. The sum of the two components (total GLP-1 concentration) reflects the rate of secretion of the L-cell. Sensitivity was below 1 pmol/l, and intra-assay CV below 5 %[25].

Antioxidant assays

The ferric-reducing activity of plasma (FRAP) assay (Benzie & Strain[26]) and the Trolox equivalent antioxidant capacity (TEAC) assay (Dragsted *et al.*[27]) were used to define changes

to plasma antioxidant capacity. For the analysis of FRAP in a microplate format, 6 μl of plasma were added with 18 μl of sterile distilled water and 180 μl of freshly prepared FRAP reagent (250 mM-sodium acetate (pH 3·6), 1·6 mM-iron (III) chloride and 0·83 mM-2,4,6-Tris(2-pyridyl)-s-triazine). Samples were incubated at 37°C for 4 min and absorbance (A_{593nm}; Dynex Technologies) recorded after 4 min and after a further 30 min at 37°C. The difference in A_{593nm} between samples and blanks was calculated and compared with a standard curve of Fe(II) standard solutions (50–1000 μM). For TEAC analysis a proprietary assay (TEAC Assay; Sigma CS0970) was used and conducted according to the manufacturer's recommendations. Briefly, 10 μl of plasma were added to 20 μl of myoglobin solution and 150 μl of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) substrate working solution. Samples were incubated for 5 min at 21°C (as optimised), the reaction stopped and the A_{405nm} recorded (Dynex Technologies). The antioxidant capacity was reported as Trolox equivalents by referral to the equation obtained from the linear regression of a Trolox standard curve.

Statistical analysis

AUC was calculated using the trapezoid approximation. For the incremental version (AUCi), only the extent of interpolated values above baseline contributed. Values obtained following the control and extract capsules were compared by paired *t* tests.

Results

The mean body weight of the volunteers remained stable during the study: 92·3 (SD 15·6) kg on visit 1 compared with 92·3 (SD 15·4) kg on visit 2. The order in which the capsules were taken (control *v.* bilberry extract) did not significantly influence any variable.

Plasma glucose

The ingestion of the bilberry extract lowered the venous plasma glucose AUCi by 18 % compared with the placebo ($P = 0·003$; Fig. 1). All eight volunteers showed a decrease in plasma glucose AUCi when taking the bilberry extract compared with the placebo (data not shown). The kinetics of glycaemia was compared for AUCi for 0–60 min and for 60–300 min but there was no difference between the bilberry extract and the placebo (Fig. 1(b)). However, the incremental plasma glucose concentrations were significantly lower at 120, 150 and 180 min after taking the bilberry extract compared with the placebo control ($P = 0·04$, 0·02 and 0·004, respectively; Fig. 1(a)). We also examined the effect of the ingestion of the bilberry extract on the glycaemic profile[28], defined as the duration of the incremental postprandial blood glucose response divided by the blood glucose incremental peak, but found no effect when compared with the placebo control (data not shown).
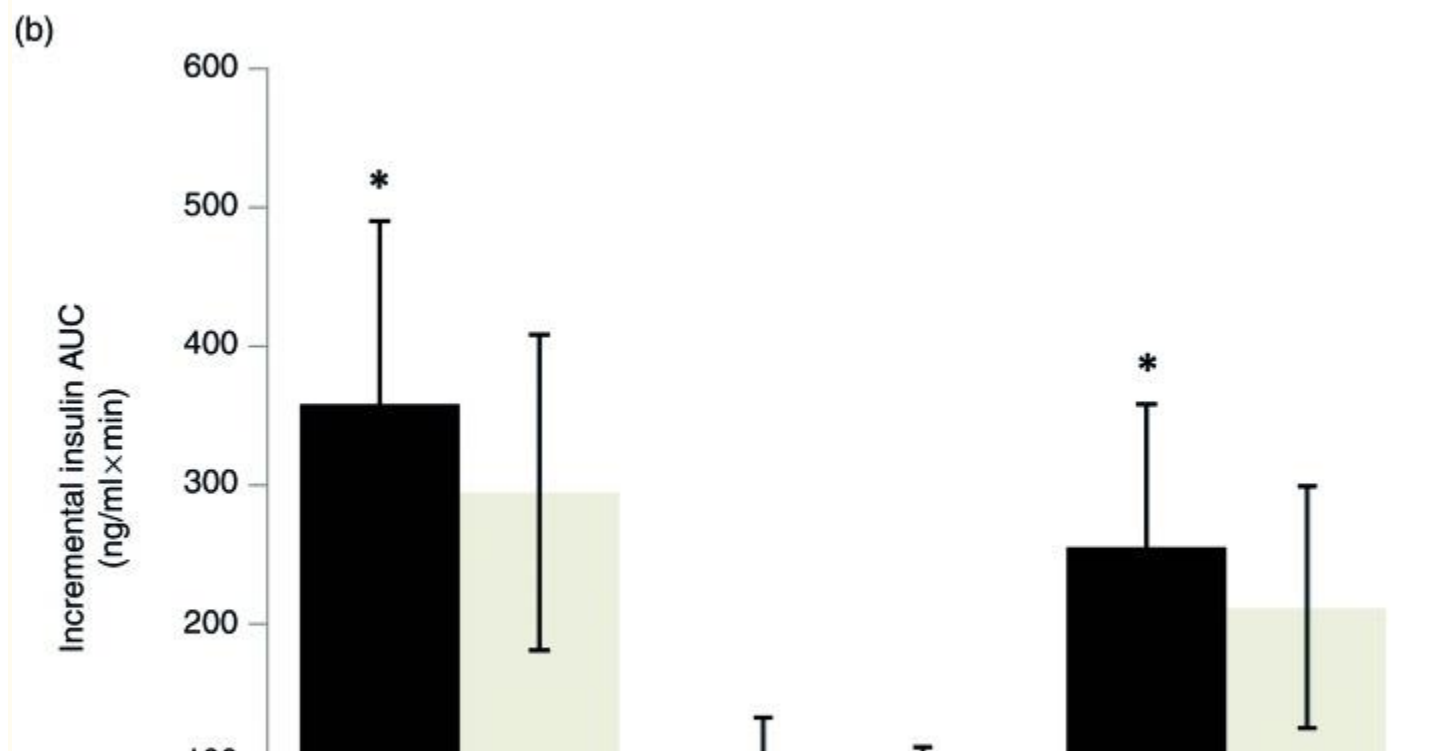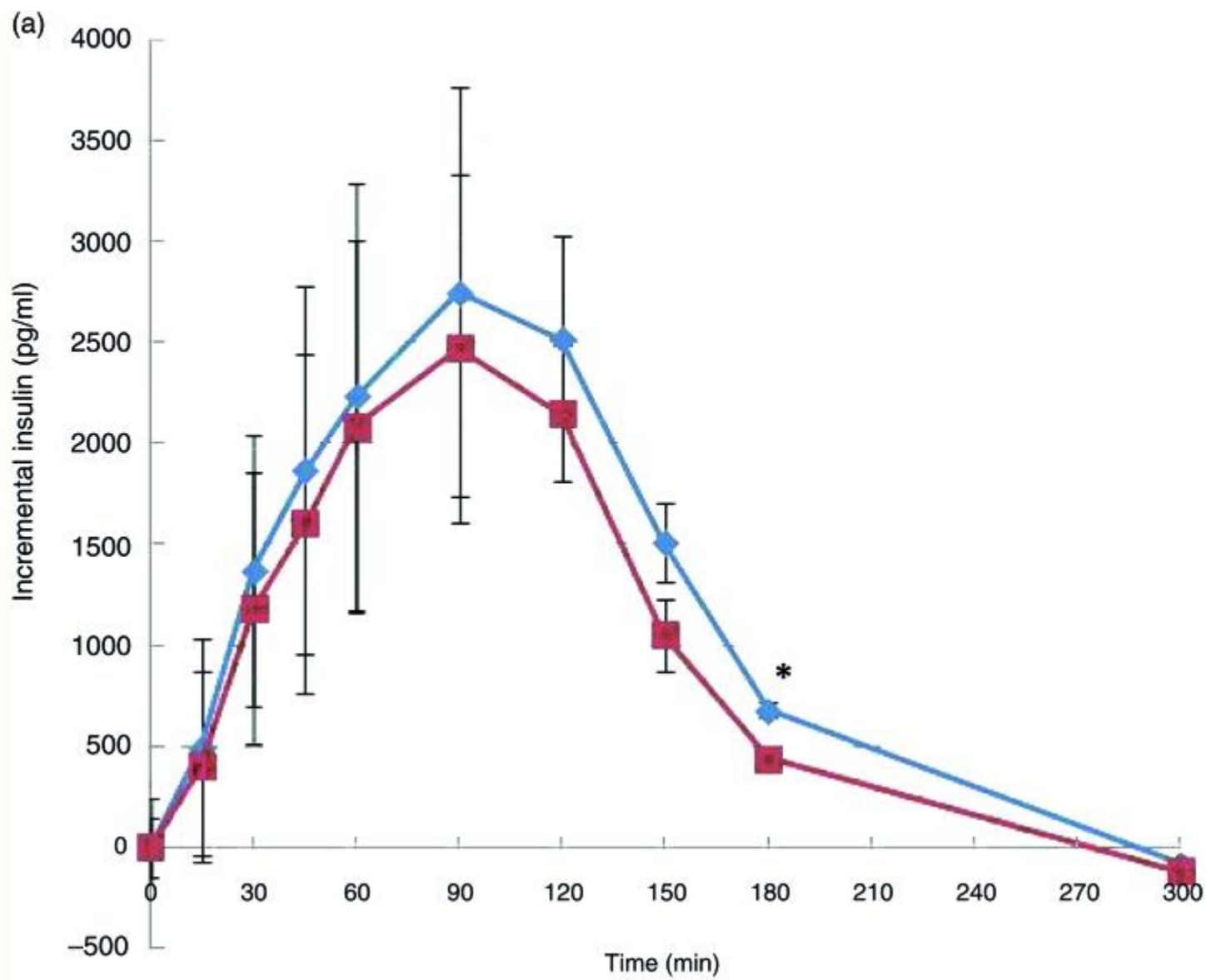
(a)

(b)
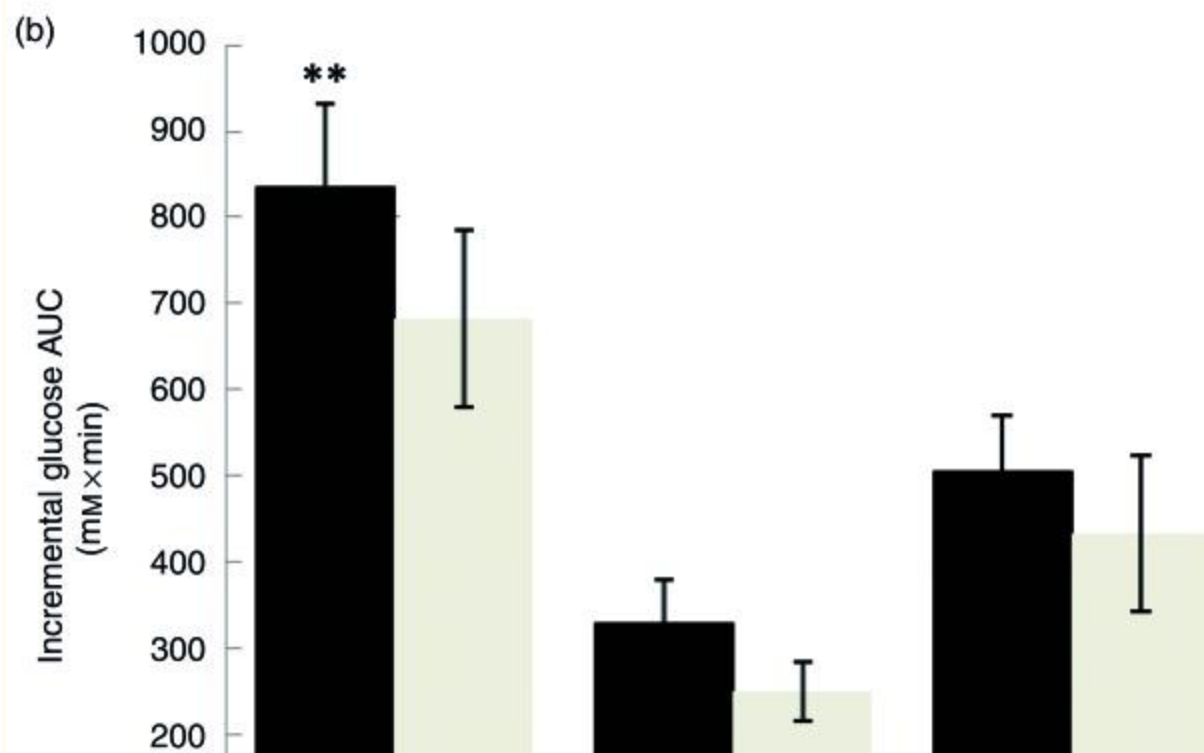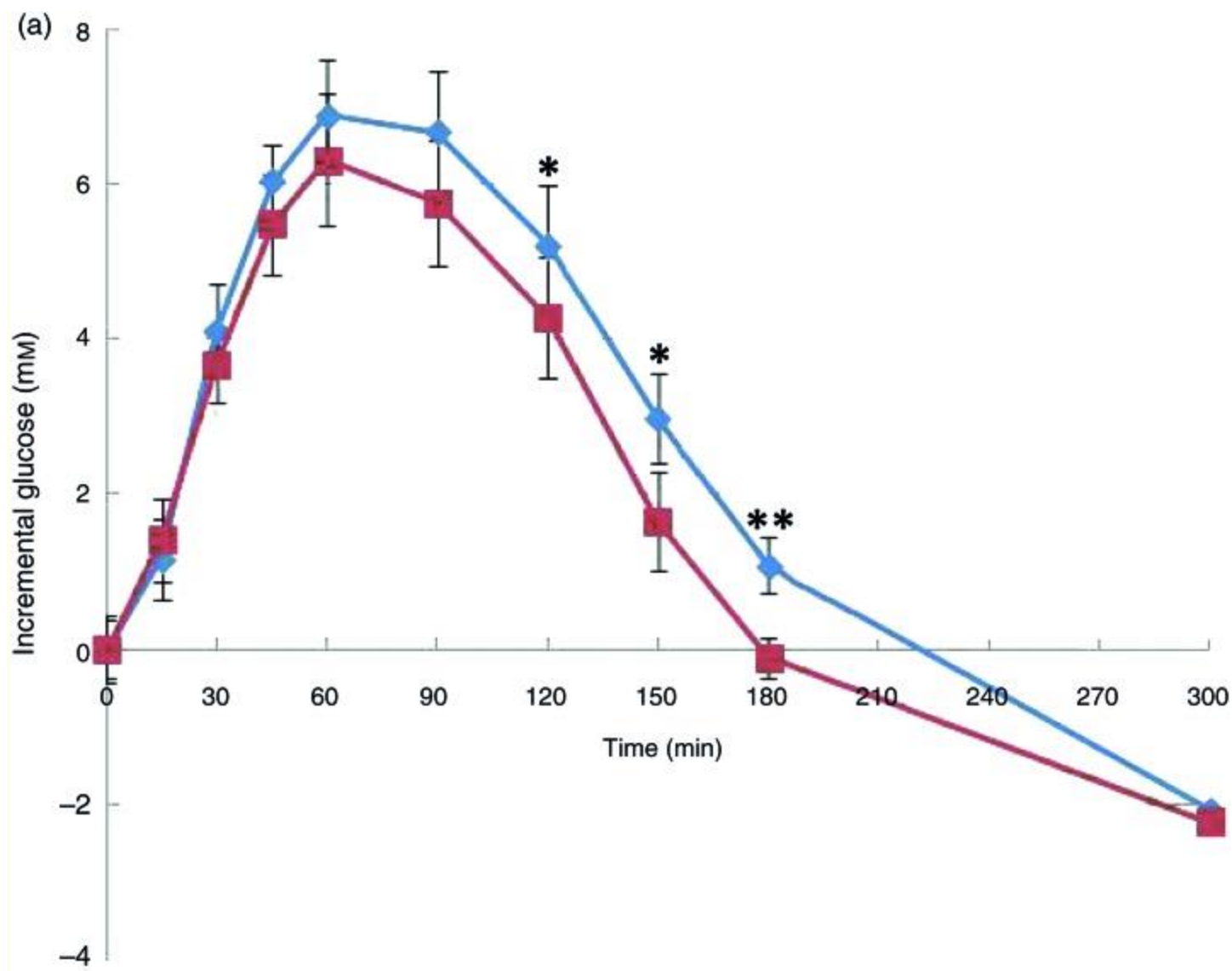
Fig. 1.

(a) Plasma incremental glucose concentrations following consumption of a glucose load with either a single placebo control (◆) or bilberry (*Vaccinium myrtillus* L.) extract (■) capsule. (b) Incremental AUC (AUCi) from 0 to 300 min, 0 to 60 min and 60 to 300 min for plasma glucose concentrations under the control (■) and bilberry extract (○) conditions. Values are means for eight subjects, with standard errors represented by vertical bars. Mean value was significantly different from that for the bilberry extract: *$P < 0.05$, **$P < 0.01$.

Plasma insulin

The ingestion of the bilberry extract lowered the venous plasma insulin AUCi by 18 % compared with placebo ($P = 0.028$; Fig. 2). All but one volunteer showed a decrease in plasma insulin AUCi when taking the bilberry extract compared with the control (data not shown). There was a 17 % decrease ($P = 0.04$) between the extract and placebo control for the time 60–300 min but not for the early postprandial phase (0–60 min; Fig. 2(b)). The incremental plasma insulin concentration was also lower at 180 min after taking the bilberry extract compared with placebo ($P = 0.04$; Fig. 2).
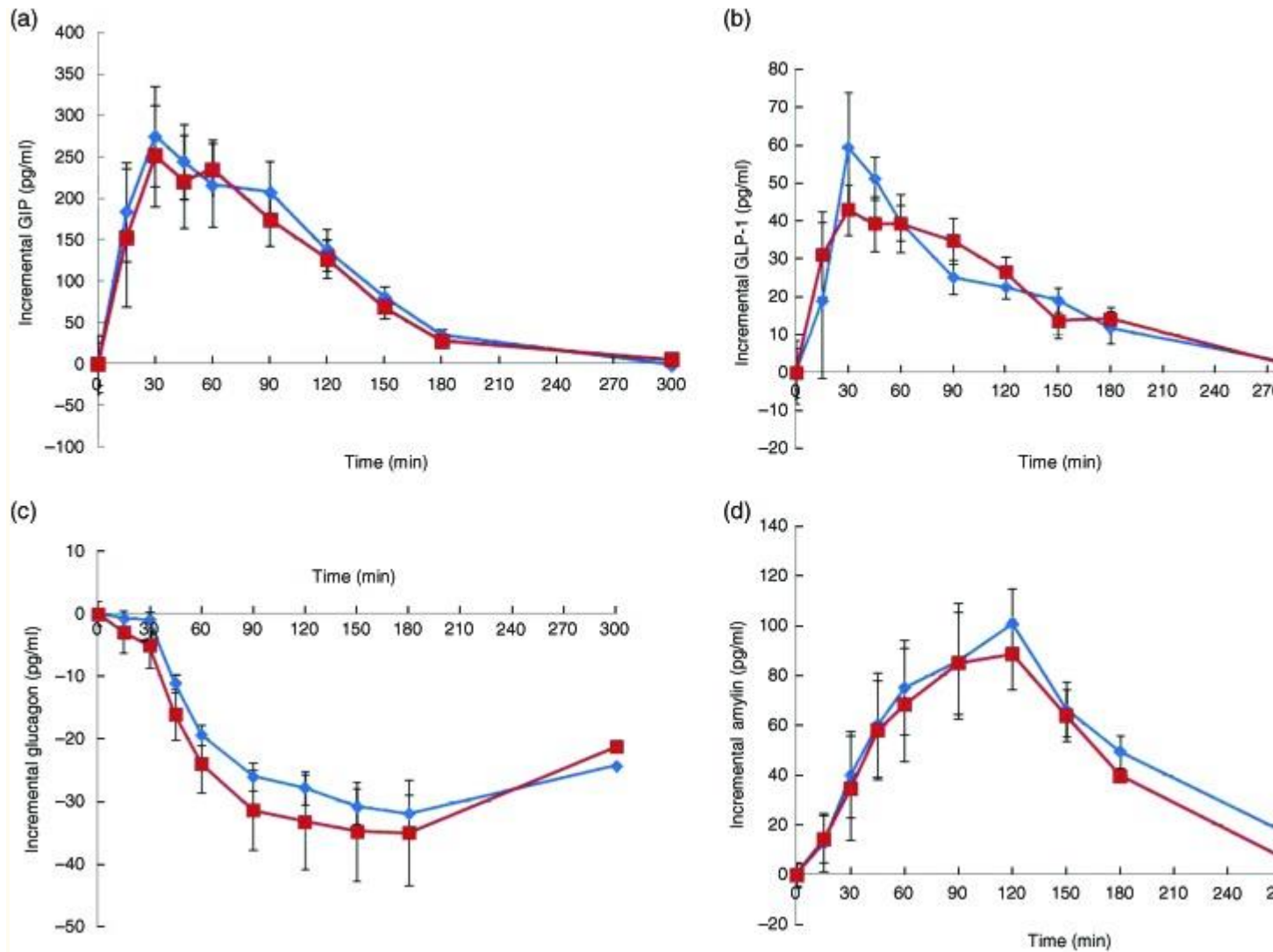
(a)

(b)

Fig. 2.

(a) Plasma incremental insulin concentrations following consumption of a glucose load with either a single placebo control (♦) or bilberry (*Vaccinium myrtillus* L.) extract (■) capsule. (b) Incremental AUC (AUCi) from 0 to 300 min, 0 to 60 min and 60 to 300 min for plasma insulin concentrations under the control (■) and bilberry extract (○) conditions. Values are means for eight subjects, with standard errors represented by vertical bars. * Mean value was significantly different from that for the bilberry extract ($P < 0.05$).

Incretin response

The impact of the bilberry extract and the placebo ingestion on the gut incretin hormones, plasma GIP and GLP-1, secreted from the intestinal mucosa, as well as glucagon and amylin secreted from the pancreas was compared at all time points. There was no difference in treatment for the AUCi for any of these hormones or for any of the individual time points compared with placebo (Fig. 3).

Fig. 3.

Plasma incremental concentrations of (a) gastric inhibitory polypeptide (GIP), (b) glucagon-like peptide-1 (GLP-1), (c) glucagon and (d) amylin from 0 to 300 min following consumption of a glucose load with either a single placebo control (♦) or bilberry (*Vaccinium myrtillus* L.) extract (■) capsule. Values are means for eight subjects, with standard errors represented by vertical bars.

Inflammatory and oxidative response

The bilberry extract had no effect on the plasma concentrations of the inflammatory adipokine MCP-1 (Fig. 4(a)) compared with the placebo control at any of the time points studied. Similarly there was no effect of the bilberry extract on the oxidative state measured by plasma FRAP (Fig. 4(b)) and TEAC (Fig. 4(c)), compared with placebo.
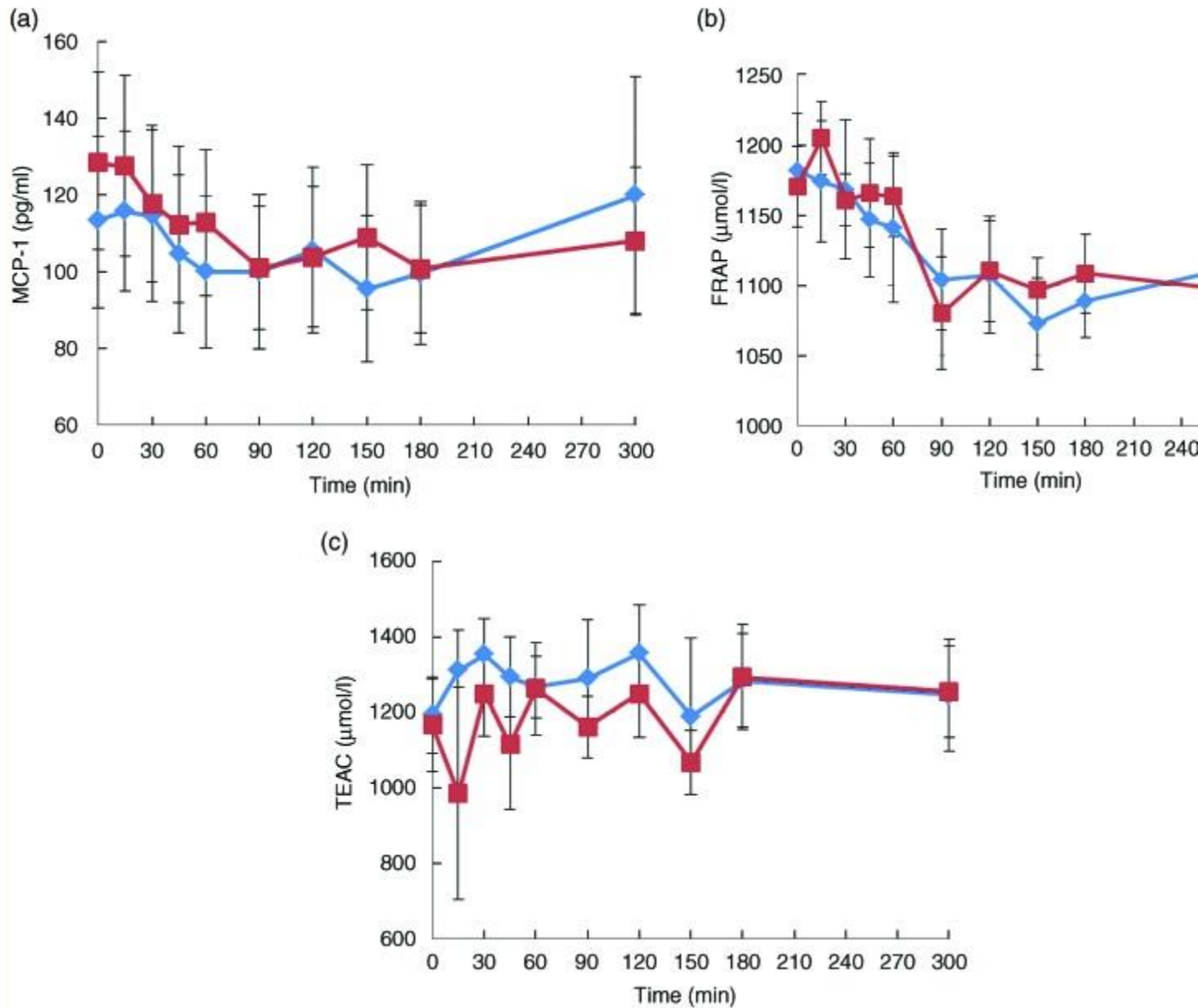
Fig. 4.

Plasma concentrations for (a) monocyte chemotactic protein-1 (MCP-1), (b) ferric-reducing ability of plasma (FRAP) and (c) Trolox equivalent antioxidant capacity (TEAC) from 0 to 300 min following consumption of a glucose load with either a single placebo control (♦) or bilberry (*Vaccinium myrtillus* L.) extract (■) capsule. Values are means for eight subjects, with standard errors represented by vertical bars.

Discussion

The present study shows that the ingestion of a capsule containing concentrated bilberry extract gives a reduced postprandial glycaemic response in volunteers with T2D controlled by diet and lifestyle alone compared with an inert placebo capsule. Given that the glucose concentrations between the volunteers taking the bilberry and control extract are different during the later time points (120, 150 and 180 min) it might be suggested that the active ingredient takes some time before it has an effect, perhaps due to digestion or where it is having its effect, for example, time to reach the gastrointestinal tract. This differs from previous studies in normal/healthy volunteers where the decrease in the plasma glucose between the volunteers taking the berries and control extract occurs at the earlier time points[, 23, 29, 30]. This may be due to differences in glucose metabolism in volunteers with T2D or differences between the studies, for example, the ingestion of a capsule may take longer to reach the gastrointestinal tract compared with a berry purée. The bilberry extract also decreased plasma insulin compared with the control in a profile that mirrors the postprandial glycaemic response. One explanation is that the decreased plasma insulin is a result of the lower plasma glucose or the volunteers become more insulin sensitive.

One study in normal/healthy volunteers that reported a mean decrease in plasma glucose after 15 and 30 min following the consumption of a commercial apple juice also observed parallel changes in the plasma concentrations of the incretins, GLP-1 and GIP[, 29]. Both these incretins are produced in the intestinal mucosa and are normally secreted when food is eaten in order to reduce glycaemic excursion by causing an increase in insulin secretion. However, GLP-1 also has other effects such as inhibiting glucagon secretion from the pancreas and by decreasing the time it takes for food to empty from the stomach. In the present study we did not find an effect of the bilberry extract on GIP, GLP-1 or glucagon. Further, we also looked at the effect of the bilberry extract on the pancreatic hormone amylin which also affects plasma glucose concentration independent of insulin secretion. Again, we did not observe any effects of the bilberry extract on plasma amylin compared with the placebo.

Bilberries are rich in anthocyanins, recognised for their ability to provide and activate cellular antioxidant protection, inhibit inflammatory gene expression, and consequently protect against oxidant-induced and inflammatory cell damage and cytotoxicity[, 2 – 4]. In light of this we investigated the effects of a bilberry extract on the inflammatory marker MCP-1 that plays a role in the recruitment of monocytes due to the low-grade inflammation associated with obesity[, 31]. However, in the present study we did not see any changes in plasma levels of MCP-1 due to the ingestion of the bilberry extract compared with the control. Similarly, we could not detect any alterations in plasma TEAC or FRAP, both markers of oxidation. It may well be that any effects of the bilberry extract on markers of inflammation and oxidation take longer than 5 h to occur. In addition, our sample size of eight volunteers was modest, and meant that we had 80 % power to detect treatment effects about 1.5 times the natural within-individual variability (SD) in outcome measurements. Therefore any negative results reported need to be viewed in this context.

It has been suggested that berry polyphenols inhibit α-glycosidase, the enzyme responsible for the digestion of sucrose to glucose in the intestinal epithelium. Two anthocyanins (cyanidin-3-rutinoside[, 32] and cyanidin-3-galactoside[, 33, 34]) have been shown *in vitro* to be inhibitors of α-glucosidase. Cyanidin-3-galactoside is present in bilberries[, 35] and cranberries[, 24], and has shown a synergistic effect with acarbose[, 34]. Acarbose is used as an inhibitor of α-glucosidase in the treatment of diabetes. Also proanthocyanidins have shown potent α-glucosidase inhibitory activity[, 36, 37]. The anthocyanins in the bilberry extract are well documented[38,39] and include both cyanidin-3-galactoside and proanthocyanidins. As the glucose load Polycal used

here for the OGTT is composed of complex carbohydrates, the reduced postprandial glycaemia in response to the ingestion of the bilberry extract may be due in part to a reduction in the breakdown of carbohydrates.

In addition there is evidence that polyphenols can affect the absorption of glucose across the intestine. This is thought to be mediated by active Na-dependent transport via Na glucose co-transporter 1 (SGLT1) and facilitated Na-independent transport via GLUT2[40]. The Na^+-dependent SGLT1-mediated glucose uptake appears to be inhibited by several phenolic acids (for example, chlorogenic, ferulic and caffeic acids)[13] as well as by glucosides of quercetin[20]. The glucose transport by GLUT2 was inhibited by the flavonols quercetin and myricetin[19,21]. These phenolic acids and flavonols with inhibitory activity against intestinal glucose uptake are common polyphenolic constituents of berries[41–43]. Thus, both a reduced breakdown of carbohydrates and lowered intestinal absorption may contribute to the improved glycaemic excursion. Further studies are needed to determine which of these mechanisms are more important *in vivo*. For example, comparison of the bilberry extract responses to Polycal *v.* a glucose OGTT would resolve how important to the breakdown of carbohydrates is in the action of the bilberry extract. Although dietary fibre has been shown to affect postprandial glucose[44], the bilberry extract used only contains 18 mg of dietary fibre (15 mg in soluble form). This small quantity is unlikely to explain the reduced glycaemic response. Future studies would also focus on dose–response effects to support the observed changes in postprandial glucose in volunteers with and without T2D.

In addition to berries, other foods rich in polyphenols have been implicated in modifying glycaemic response. Various studies on the effect of coffee have suggested that the chlorogenic acid in coffee might have an antagonistic effect on the transport of sucrose[45] and attenuate the glycaemic response to sucrose[46]. However, as far as we know, none of these studies has shown a change in the AUCi values for glucose or insulin in response to the polyphenols compared with the control.

In conclusion, to our knowledge this is the first report showing that ingestion of a concentrated bilberry extract at amounts that can be easily tolerated produces a reduced AUCi postprandial glycaemia and insulinaemia in volunteers with T2D. The probable mechanism(s) for the reduced glycaemic response are reduced rates of carbohydrate digestion and/or absorption. The use of berry polyphenols as phytochemicals capable of lowering the glycaemia response to carbohydrates not only in subjects with diabetes but also in those with impaired glucose tolerance control may prove to be useful in helping control blood sugar. Such a strategy could complement the effectiveness of other lifestyle interventions such as avoidance of overweight and the need to take regular exercise.