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Acute Effects of a Spinach Extract Rich in Thylakoids on Satiety: A Randomized Controlled Crossover Trial

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Abstract

Objective—By retarding fat digestion, thylakoids, the internal photosynthetic membrane system of green plants, promote the release of satiety hormones. This study examined the effect of consuming a single dose of concentrated extract of thylakoids from spinach on satiety, food intake, lipids, and glucose compared to a placebo

Design—Sixty overweight and obese individuals enrolled in a double blind randomized cross-over study consumed the spinach extract or placebo in random order at least a week apart. Blood was drawn for assessments of lipids, and glucose before a standard breakfast meal, followed four hours later by a 5 g dose of the extract and a standard lunch. Visual analog scales were administered before lunch and at intervals until an *ad libitum* pizza dinner served four hours later. Two hours after lunch a second blood draw was conducted. Mixed models were used to analyze response changes.

Results—Compared to placebo, consuming the spinach extract reduced hunger ($p < 0.01$) and longing for food over two hours ($p < 0.01$), and increased post-prandial plasma glucose concentrations ($p < 0.01$). There were no differences in plasma lipids, and energy intake at dinner; but, males showed a trend towards decreased energy intake ($p = 0.08$).

Conclusions—At this dose, the spinach extract containing thylakoids increases satiety over a two hour period compared to a placebo. Thylakoid consumption may influence gender specific food cravings.

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Conflicts of Interest:

Dan Edwall works with Greenleaf Medical AB and Charlotte Erlanson-Albertsson is the Scientific advisor for Greenleaf Medical AB and part owner of Thylabisco AB, a research company. Candida Rebello, Jessica Chu, Robbie Beyl, and Frank Greenway have no conflict of interest.

Introduction

Appetite reflects a complex interaction among the external environment, the behavioral profile, and subjective states as well as the storage and utilization of energy [1]. Thus, the initiation and termination of ingestive behavior has both metabolic and non-metabolic components. An eating episode can be sparked by metabolic need, hedonic drive, or an interaction between the two. A neural network sensitive to energy status signals has been identified as the homeostatic control system for the regulation of food intake and energy balance [2]. The system is powerfully designed to protect the lower limits of adiposity by modulating the processing of cognitive and reward functions [2]. However, in the modern world, humans, often eat in the absence of any metabolic feedback requiring replenishment of diminished reserves. This non-homeostatic or hedonic eating involves cognitive, reward, and emotional aspects. Cues that have a reward associated with them, once learned, trigger motivational wanting to secure these rewards [3].

The photosynthetic membrane of chloroplasts consists of a system of paired membranes, the thylakoids. The thylakoid membrane system forms a physically continuous three-dimensional network that encloses an aqueous space, which is the thylakoid lumen [4]. Approximately 70% of the thylakoid mass consists of the membrane proteins and their bound pigments such as chlorophyll, carotenes, and xanthophylls. The remaining 30% largely consists of the membrane lipids such as galactolipids, phospholipids, and sulfolipids [5].

Thylakoid membranes are found in green plants such as spinach, A patented [6] extract of spinach containing significant amounts of thylakoids has been shown to have an inhibitory effect on lipase activity [7]. This inhibition is largely mediated by the protein fraction [7]; but, the membrane galactolipids may also have a role [8]. Delayed fat digestion increases the production of the satiety hormones cholecystokinin [9],[10] and glucagon-like peptide-1 (GLP-1) [11] as has been demonstrated in human trials. Additionally, in humans [9] and pigs [12] ingestion of the extract has been shown to suppress the hunger hormone ghrelin. Among the gut hormones involved in appetite regulation GLP-1 in particular has been associated with the regulation of reward induced eating behavior [13]. Thylakoid-induced increase in the precursor for enterostatin a peptide involved in appetite suppression and thermogenesis has also been demonstrated [7].

In studies with rats and mice [14, 15], significant reductions in body weight and percent body fat occurred when the diet was supplemented with the spinach extract containing thylakoids. In overweight women, a breakfast meal supplemented with 3.7 g or 7.4 g of the spinach extract suppressed subjective hunger compared to a control in a cross-over study with no statistical difference between the two doses [10]. Overweight women, consuming 5 g of the spinach extract for three months demonstrated 43% greater loss of body weight compared to a placebo The women also exhibited a decreased the urge for sweet and chocolate by 95% and 87%, respectively. The reduced urge for sweets was significant after a single dose and was sustained throughout the study demonstrating that no tolerance developed during the 3 months of daily usage [11]. Further, unlike pharmaceutical lipase inhibiting drugs, the thylakoids temporarily delay but do not prevent fat digestion. Thus, the

excretion of undigested fat which is an unpleasant side effect of lipase inhibitor drugs is avoided.

In this study subjective satiety ratings and food intake following a single administration of thylakoids from spinach leaves or a placebo, were measured. The hedonic and reward responses to food related stimuli were also evaluated. Plasma glucose and lipid concentrations were measured. It was hypothesized that thylakoid supplementation would produce an increase in satiety that would be accompanied by the appropriate changes glucose and lipid measures.

Subjects and Methods

Participants

Sixty overweight or obese males and females between the ages of 18 to 65 years were recruited from Baton Rouge and the surrounding areas to evaluate the effect of thylakoid supplementation on appetite, satiety, and food intake. Inclusion criteria specified that all subjects should have a body mass index between 25 and 35 kg/m^2 and a waist circumference over 35 inches. Subjects were excluded if: (i) they had existing medical conditions or were taking medications that could influence their appetite, food absorption, body weight, or mood, and (ii) were currently or during the previous two months on a low calorie diet.

All subjects participated in an initial screening that involved measurement of body weight, height, waist circumference, and vital signs (blood pressure and pulse rate). At screening, health was assessed through the administration of a medical screening questionnaire and subjects underwent a medical examination to confirm their medical suitability for participation in the study. Sixty subjects passing the screening were randomized to two groups of 30 each. This study was approved by the Institutional Review Board of the Pennington Biomedical Research Center, Baton Rouge, where the study was conducted. Participants provided written informed consent. The trial was registered on ClinicaTrials.gov with registration number NCT01919814.

Design

The study followed a double blind placebo-controlled randomized cross-over design. Each participant was tested on two days. On one occasion the test product consisted of a concentrated extract of thylakoids from spinach (Appethyl™, Green Leaf Medical, Stockholm, Sweden) and on the other occasion the test product consisted of the placebo served in random, balanced order. At the first test visit, subjects arrived at the center after a 12 hour overnight fast, and having avoided strenuous exercise for 24 hours prior to arrival. Body fat was measured using bioelectrical impedance analysis (Tanita Corporation of America Inc. Arlington Heights, Illinois) and subjects were allowed to rest for a few minutes prior to having a blood sample drawn for assessment of plasma triglycerides (TG), total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) free fatty acids (FFA), highly sensitive C-Reactive Protein (hsCRP), and glucose. After eating a standardized 300 kcal breakfast meal they were advised to remain in the dining area of the metabolic kitchen and refrain from any food or snack

consumption until lunch. Before eating lunch subjects were required to rate their satiety using electronic visual analog scales (VAS) [16, 17]. They then consumed the spinach extract or placebo prior to being served a standardized 750 kcal lunch meal.

Breakfast and lunch were supervised to ensure that the entire meal was eaten. Visual analog scales assessing satiety were administered at 30, 60, 120, and 240 minutes after the start of the lunch meal. Two hours after lunch a second blood draw was conducted to obtain postprandial measures of TG, total cholesterol, HDL-C, LDL-C, FFA, hsCRP, and glucose. Liking and wanting [18] were assessed four hours after lunch followed by an *ad libitum* dinner meal consisting of pizza. The pizza meals were pre-weighed and participants were presented with a meal that was in excess of what they could possibly eat. They were told to eat to satisfaction over 20 minutes, after which the remains of the meal were weighed. Subjects were required to remain at the Center between lunch and dinner. The food intake at dinner was determined by subtracting the weight of the uneaten food from its original weight. The energy and macronutrient intakes were calculated using product information.

Study Products and Meals

Five grams of the spinach extract were well mixed with two fluid ounces of diet Blueberry Pomegranate Juice (Ocean Spray) and one teaspoon of corn oil. For the placebo 2.5 g of cornstarch, 2.5 g of All-purpose flour, 5 g of glycerin, one teaspoon of corn oil, and food coloring were well mixed with two fluid ounces of diet Blueberry Pomegranate Juice. The test products were prepared and served immediately. A description of the standardized breakfast and lunch meals is provided in Table 1. Pepperoni pizza (Tombstone™ Original, Glendale, CA) was served at the *ad libitum* diner meal.

Subjective Satiety, Liking, and Wanting Assessment

Hunger, fullness, desire to eat, prospective intake, satisfaction, thirst, and appetite for sweet, salty, and savory foods were assessed using VAS. Liking refers to the affective reaction reflecting the hedonic response to a food and is the result of a central process that integrates the sensory properties as well as the individual's physiological state and associative history. Using the method developed by Finlayson *et al* [18] 'Liking' was measured through VAS ratings associated with food image stimuli varying across the dimensions of fat (high or low) and taste (sweet or savory). Thus, using 16 pictures, the foods could be arranged into combined categories where four were high fat sweet, four were high fat savory, four were low fat sweet, and four were low fat savory foods. These foods could also be arranged into generic groups with eight foods in each of the categories high fat, low fat, sweet, and savory. The images were relatively similar in size and in color. The order in which images were presented to subjects was randomized by time point using a random number generator. Each image was paired with the following questions administered using VAS: (i) How pleasant would it be to experience a mouthful of this food now? (ii) How often do you eat this food? (iii) How pleasant do you find this food? (iv) How much do you want some of this food now?

Wanting refers to an underlying implicit and objective drive process that mediates an intent or desire to consume a food [18]. Called incentive salience, it reflects a motivating desire

that the brain attributes to reward-predicting cues [19]. Wanting was measured by presenting an image of a food stimulus paired with another image of a different food stimulus and subjects were asked to select the food they "most want to eat now". The images used in the 'Wanting' procedure were the same images used for the 'Liking' procedure and each image in the set was uniquely paired with every possible image outside of its food category. For instance, a high fat sweet food was paired with every low fat sweet, high fat savory, and low fat savory food. Thus, using 16 images, 96 pairs of food images were presented and participants had to choose one of the two foods presented imagining they could eat as much or as little of that food as they wanted.

Statistical Analyses

To analyze the differences in the VAS ratings of satiety between the spinach extract and placebo conditions, a linear mixed model was used to estimate how the ratings for each of the questions changed over time. The covariates in the model were time and cross-over order effect, and an unstructured covariance matrix was used to model the relation between time points for each subject. The responses defined as change from baseline (the measure just prior to lunch) included measures at 30, 60, 120, and 240 minutes following the lunch meal. T-tests based on the least squares means from the model were used to determine differences between the two conditions.

The hedonic impact of each food category was assessed by calculating the mean liking scores and the differences in scores between the test and the control conditions, for each of the questions asked. The differences in wanting were assessed by calculating the mean frequency scores for food selection, in each food category. Food intake was evaluated as the difference in mean scores between the between the spinach extract and placebo conditions using a linear model that adjusted for a crossover effect. A sub-analysis based on gender was also performed. Metabolic parameters were assessed as change from baseline using a linear model adjusted for a crossover effect. T-tests were used to determine differences between the two conditions. All values are expressed as least squares means ± standard error. Statistical analyses were performed using SAS (version 9.4, SAS Institute, Cary, NC).

Results

Sixty participants, 30 males and 30 females were enrolled in the study. One female participant had a severe headache which the participant related to the temperature in the testing room. It was treated as a serious adverse event and the available data relating to this participant was not included in the analysis. Additionally, two other participants were dropped from the study due to one being diagnosed with diabetes and the other having a schedule conflict. Descriptive characteristics of participants included in the analyses are presented in Table 2.

Visual Analog Scales

Analysis of individual satiety ratings revealed that compared to the placebo, consumption of the spinach extract significantly increased fullness ($p=0.04$), and reduced hunger ($p<0.01$), longing for food ($p<0.01$), and prospective intake ($p=0.01$) over the two hour period

following lunch (Fig. 1 A, B, C, D), while satisfaction was not significantly different (Fig 1 E). In addition, consumption of the spinach extract reduced the desire for something salty ($p<0.01$), desire for something savory ($p<0.01$), and thirst ($p<0.01$) over the two hour period following lunch, compared to the placebo (Figure 2 A, B, C); however, the desire for something sweet was not significantly different (Figure 2 D). At four hours thirst was still suppressed after consuming the spinach extract ($p=0.02$), while there were no significant differences between the two conditions for the other ratings.

Intake at Dinner Meal

There were no significant differences in food intake at the dinner meal served four hours after lunch between the two conditions. The total weight of food consumed and energy intake were also not significantly different. Males exhibited a trend towards decreased consumption, reducing their energy intake by 49.3 g (125.7 kcal, $p=0.08$); whereas, females increased their intake by 3.2 g (8.1 kcal, $p=0.85$).

Liking and Wanting

There were no significant differences in the mean scores for all the questions asked in each food category, in the test to assess differences in liking between the spinach extract and placebo conditions. No significant differences were found in the mean scores for frequency of choosing foods in each food category, in the test to assess the differences in wanting between the two conditions.

Metabolic Parameters

There were no differences in the plasma concentrations of TG, total cholesterol, HDL-C, LDL-C, FFA, and hsCRP. However, the difference in fasting and post-lunch plasma glucose concentrations was greater ($p<0.01$) in the spinach extract condition compared to the placebo (Figure 3).

Discussion

Compared to a placebo, a single supplementation with 5 g of thylakoids increased satiety measured subjectively over two hours. This was accompanied by a greater increase in the post-prandial plasma glucose response Satisfaction, which introduces a hedonic component into the measurement of satiety [20], was included to determine if satiety measures were judged from a comparable baseline during the repeated testing. As expected, it was not significantly different between the conditions. However, over a four hour period the differences in satiety measures were no longer significant. Differences in energy intake at the *ad libitum* meal and in the liking and wanting components of food reward measured at four hours after consuming thylakoids were also not significant between the two conditions.

The spinach extract (Appethyl™) contained concentrated thylakoids extracted from the choloroplasts of spinach leaves. By interacting with lipids and retarding fat digestion thylakoid membranes promote the release of satiety hormones such as cholecystokinin and reduce the hunger stimulating hormone ghrelin [9]. *In vitro*, thylakoid membrane proteins bind to lipid droplets at the oil-water interface as well as to the lipase-colipase complex [7].

Thus, inhibition of lipolysis could occur through blocking the access of the lipase-colipase complex to the fat droplet or blocking of the active site of the lipase-colipase complex and preventing the enzyme complex from coming in contact with the fat droplet [7]. It has also been suggested that the thylakoid membrane lipid digalactosyldiacylglycerol, with its large polar head groups may shield colipase and sterically hinder the formation of the lipase-colipase complex necessary for lipolysis [8].

Thylakoid membranes effectively suppressed lipolytic activity in a dose dependent manner during *in vitro* hydrolysis of an emulsion of TG dispersed in bile salts [7]. Suppression of lipase-colipase activity stimulates a compensatory increase in endogenous secretion and production of lipase and colipase. This compensatory release has been suggested as a mechanism for increasing the satiety hormone cholecystokinin and the appetite suppressing peptide enterostatin in response to thylakoid supplemented high fat meals [7].

In the present study, there was no difference in the blood lipid concentrations between the thylakoid and placebo conditions at two hours. Following a fat containing meal, TG reach their maximal concentrations three to four hours after consuming a single meal and return to baseline within six to eight hours [21–24]. Following sequential meals the plasma appearance of TG peaks at 60 minutes and thereafter falls up to 240 minutes [23]. Free or non-esterified fatty acid concentrations fall in response to a single meal and rise thereafter peaking between four and five hours post-prandially [21–24]. Following two sequential meals FFA increase within 30 to 60 minutes and thereafter fall [23]. Based on the kinetics of fat absorption it is difficult to determine if there was a delay in lipid absorption without repeated testing before and after the two hour period.

The prolonged presence of nutrients in the gastrointestinal tract as well as cholecystokinin release prompts the release of GLP-1 from the distal regions of the intestine [11]. Supplementation with 5 g of the spinach extract significantly increased the difference between the pre- and post-prandial GLP-1 levels after a single instance as well as following 12 weeks of supplementation, compared to a placebo treated group [11]. GLP-1 acts on the reward system [13]; hence, it may provide an explanation for the decrease among males, in energy intake at the dinner meal served four hours after consuming the spinach extract. Males exhibit a yearning for savory foods [25–27], whereas females prefer high fat sweet foods such as chocolate [25, 27, 28]. Although not significant, which may perhaps be because the study was not powered to detect differences in food intake by gender, males reduced their intake of savory food (pizza) by 126 kcal after consuming the spinach extract.

It has been demonstrated that thylakoids consumption reduces the urge for chocolate among women [11]. However, in the present study the option of sweet foods was not presented when food intake was evaluated at the *ad libitum* dinner meal. This resulted in an overall non-significant change in energy intake between the spinach extract and control conditions. The absence of a sweet food at the dinner meal may also explain why desire to eat something savory decreased with supplementation of the spinach extract; but, desire to eat something sweet was not different between the conditions. Nevertheless, it is estimated that weight gain in 90% of the adult population in the US is due to a positive energy balance of

100 kcal/day or less [29]. Thus, a reduction in energy intake of 126 kcal may have clinical relevance.

The increase in plasma glucose concentrations at the two hour time point may be explained by a decrease in insulin secretion. In humans, a single thylakoid-enriched high fat meal increased the satiety hormones cholecystokinin, and leptin while reducing levels of insulin and the hunger-stimulating hormone ghrelin [9]. A reduction in insulin secretion by supplementation with thylakoids has also been demonstrated in a porcine study [12]. In a previous study [10], a high carbohydrate meal (71% of energy) supplemented with the spinach extract resulted in a tendency towards higher plasma glucose from 90 minutes following the meal, compared to a control meal. The greatest difference occurred at 120 minutes; although, over four hours the differences were not significant. In that study, the trend towards increased blood glucose was accompanied by suppressed hunger from 180 minutes.

In the present study, significantly higher plasma glucose concentrations coincided with greater satiety. Increasing extracellular glucose concentration inhibits the orexigenic agouti-related peptide/neuropeptide Y-expressing neurons and stimulates the anorexigenic proopiomelanocortin and cocaine-and amphetamine-related transcript expressing neurons [30]. Thus, the greater increase in plasma glucose concentration two hours post-lunch may in part explain the increase in satiety following supplementation with the spinach extract compared to the placebo. Longer term (90 days, 5g/day) consumption of thylakoids decreased body weight, but had no effect on plasma glucose concentrations. There was a significant reduction in blood glucose and insulin on day one, whereas on day 90, there were no differences in blood glucose or insulin concentrations between control and treated groups [11]. Thus, the decrease in insulin secretion and rise in two hour post prandial glucose concentrations does not seem to adversely affect glycemic or insulinemic control over time. Moreover, thylakoids administered with a low-carbohydrate breakfast had no effect on serum glucose concentration at two hours [9]. Therefore, the effect of thylakoids on glucose homeostasis may depend on the carbohydrate content of the meal. The glycemic and insulinemic effect may also dissipate before 90 days; however, more studies are needed to establish these relationships.

The main limitation of this study is that metabolic parameters were assessed at a single time point. Frequent and more prolonged testing would have provided a better determination of the physiological mechanisms influencing satiety. Moreover, insulin and gut hormones were not measured. Further, males and females differ in their food cravings and the *ad libitum* meal did not cater to both gender preferences. A larger sample of males and females than the study provided would have helped to clarify potential mechanisms relating to the effects of thylakoids on the reward system.

Conclusions

Consistent with previous research a single meal supplemented with 5 g of a concentrated extract of thylakoids from spinach increases satiety over the two hour period following consumption. Thylakoids supplementation may influence food cravings by acting on the

reward system, thereby offering a novel way to address a positive energy balance in a manner that is minimally burdensome on the consumer. Gender based studies of the effect of thylakoids consumption on appetite regulation, employing appropriate sample sizes, are needed.

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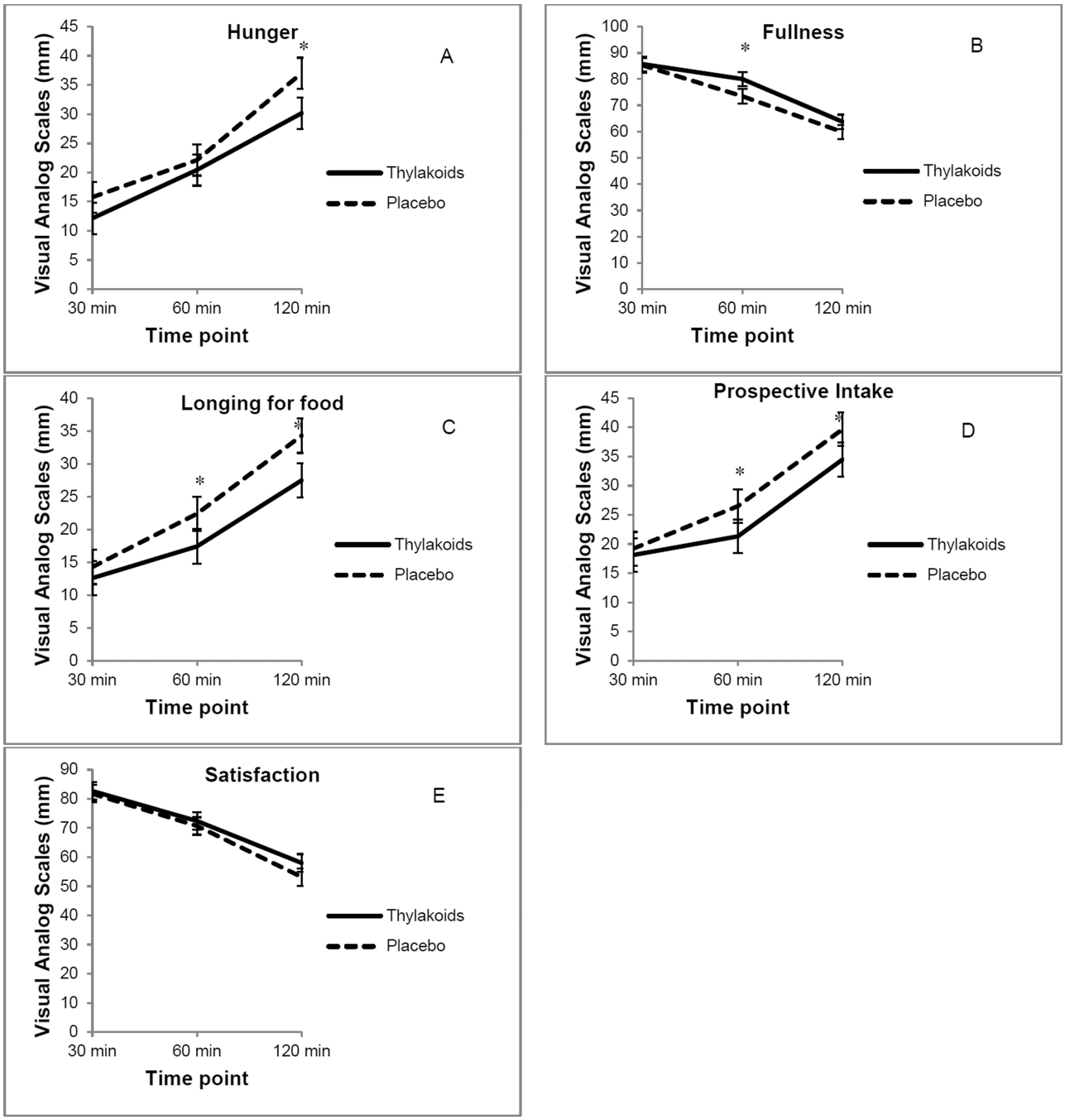

Figure 1.

A, B, C, D, E Visual analog scale ratings for satiety (n = 59) over two hours after consuming the spinach extract or a placebo. **(A)** Hunger: Overall differences, 4.0720 mm ± 1.52 ($p < 0.01$), *120 min ($p < 0.01$) **(B)** Fullness: Overall differences, 3.62 mm ($p = 0.04$), *60 min ($p = 0.03$) **(C)** Longing for food: Overall differences, 4.50 mm ± 1.32 ($p < 0.01$), *60 min ($p < 0.03$) and *120 min ($p < 0.01$). **(D)** Prospective intake: Overall differences, 3.83 mm ± 1.35 ($p < 0.01$), *60 min ($p = 0.03$) and *120 min ($p = 0.03$). **(E)** Satisfaction: Overall differences were not significant. Values are mean ± standard error

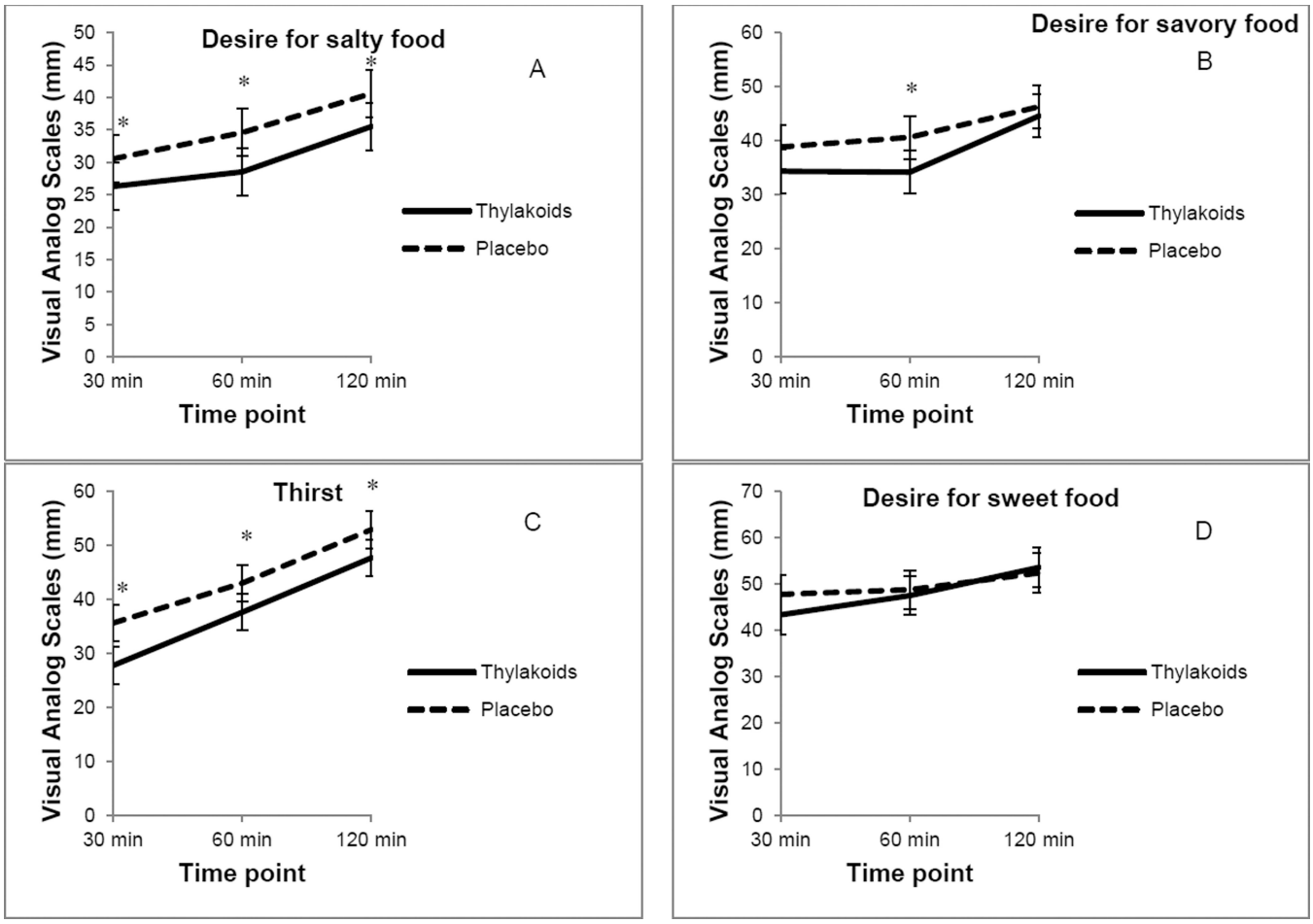

Figure 2.

A, B, C, D Visual analog scale ratings for satiety (n = 59) over two hours after consuming the spinach extract or a placebo. Values are mean ± standard error **(A)** Desire for salty food: Overall differences, 5.14 mm ± 1.09 ($p < 0.01$), *30 min ($p = 0.02$), *60 min ($p < 0.01$), and *120 min ($p < 0.01$). **(B)** Desire for savory food: overall differences, 4.22 mm ± 1.4 ($p < 0.01$) *60 min ($p < 0.01$). **(C)** Desire for sweet food: Overall differences were not significant. **(D)** Thirst: Overall differences, 6.16 mm ± 1.46 ($p < 0.01$) * 30 min ($p < 0.01$), *60 min ($p = 0.03$), and *120 min ($p = 0.04$).

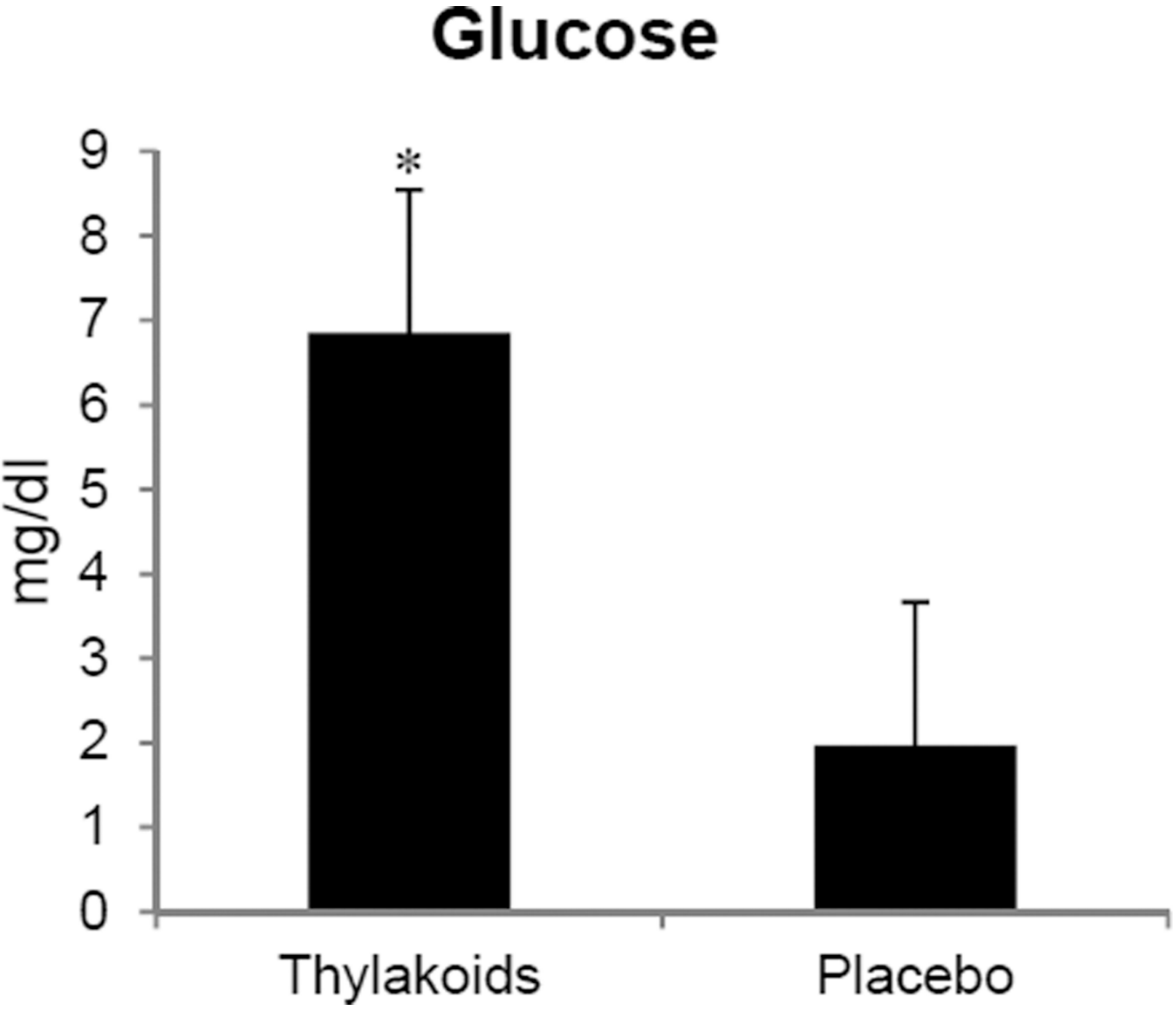

Figure 3.

Difference in fasting and two hour post-lunch plasma glucose concentrations between the spinach extract and placebo conditions. *Significantly different at $p < 0.01$. Values are mean ± standard error.

Table 1

Energy and Macronutrient Content of Standard Lunch and Breakfast Meals

Food	Energy (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
Breakfast				
Whole Wheat Bread	133.0	4.9	22.6	1.6
Butter, Salted	22.0	0.0	0.0	2.4
Cheese, Cheddar	52.0	3.2	0.2	4.3
Extra Lean Ham	43.0	5.7	0.7	1.4
Egg, Scrambled	50.0	3.3	0.7	3.7
Water	0.0	0.0	0.0	0.0
Total	300.0	17.1	24.2	13.4
Lunch				
Oven Roasted Turkey	75.0	15.0	1.5	0.8
Cheese, Swiss	120.0	8.5	1.7	8.8
Whole Wheat Bread	165.0	6.0	28.0	2.0
Lettuce	4.0	0.3	0.8	0.1
Tomatoes	5.0	0.3	1.2	0.1
Mayonnaise	115.0	0.2	0.6	12.5
Mustard, Yellow	7.0	0.4	0.5	0.4
Fritolay™ Sunchips	209.0	3.4	28.6	9.0
Fruit Salad	50.0	0.0	15.0	0.0
Water	0.0	0.0	0.0	0.0
Total	750.0	34.1	77.9	33.7

Table 2

Characteristics at baseline of 59 participants who were included in the mixed model analysis

	Mean	SD[a]	Range
Age (years)	35.3	12.4	18 – 64
Height (cm)	171.4	9.6	153.2 – 194.5
Weight (kg)	88.0	10.9	67.3 – 114.0
BMI (kg/m^2)	29.9	2.6	25.2 – 34.5
Percent Body Fat	35.1	8.3	20.4 – 47.8
Waist Circumference (cm)	97.8	6.4	88.9 – 114.3

[a]Standard Deviation