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Physiological Properties of Dietary Ellagitannin-Rich Preparations Obtained from Strawberry Pomace Using Different Extraction Methods

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The objective of this study was to establish the composition of strawberry preparations rich in ellagitannins obtained using water or acetone extraction (EF and EP preparation, respectively). Then, biological effect of these extracts was assessed in 4-wk nutritional experiment on Wistar rats. The preparations were applied in cholesterol-containing diets that had equal content of ellagitannins (0.03%). To measure animals response, parameters describing the caecal fermentation (ammonia and short-chain fatty acid concentrations, bacterial enzymes activity), blood serum lipoprotein profile, and TBARS content in selected tissues (heart, liver, kidney) were assessed. Apart from polyphenols, including ellagitannins (7.8 and 7.1%, respectively), the EF preparation contained high quantities of soluble dietary fibre and other carbohydrates (33.3 and 38.9%, respectively), whereas the EP preparation was characterised by 58.9% content of ellagitannins, no dietary fibre and a high content of proanthocyanidins (16.9%). In comparison to EF group, the dietary treatment with EP had a stronger effect on caecal environment as manifested by decreased digesta bulk, β-glucuronidase activity and total short-chain fatty acid concentration ($P<0.05$ vs. group C without supplementation). Both preparations lowered lipaemia and glycaemia. It could be concluded that more efficient acetone extraction of strawberry pomace increased the content of both ellagitannins and proanthocyanidins in the polyphenolic preparation, which caused a stronger inhibiting effect on caecal fermentation processes and at the same time lowered blood triacylglycerols and glucose level. Considering the equal content of ellagitannins in both supplemented diets, it may be speculated that the above effects were due to the presence of proanthocyanidin fraction.

INTRODUCTION

Fruits of strawberry *(Fragaria x ananassa* Duch.) are characterised by a high content of polyphenols, vitamin C, micro- and macro-elements and dietary fibre [Giampieri *et al*., 2012; Da Silva Pinto *et al*., 2008]. The main phenolic compounds of strawberry are anthocyanins [Giampieri *et al*., 2012; Oszmiański *et al*., 2009; Aaby *et al*., 2012], proanthocyanidins [Oszmiański *et al*., 2009; Buendía *et al*., 2010] as well as ellagic acid and ellagitannins [Giampieri *et al*., 2012; Da Silva Pinto *et al*., 2008]. The content of ellagitannins in strawberries depends on the variety and ripeness of fruits and reaches 637 mg/kg on average [Gasparotti *et al*., 2013]. Ellagitannins – high contents of which may be found in fruits of plants of the family *Rosaceae* (strawberries, raspberries and blackberries) – are high-molecular esters of a monosaccharide (usually β-D-glucose) and a few residues of hexahydroxydiphenic acid [Okuda *et al*., 2009]. They are characterised by a complex structure that has not been entirely explored [Gasparotti *et al*., 2013]. Agrimonine, having a rare in nature structure of a α-galloyl-HHPP-glucose dimmer, is the main ellagitannin of strawberry fruit [Vrhovsek *et al*., 2012] and pomace [Sójka *et al*., 2013]. Ellagitannins and ellagic acid are metabolised by intestinal microbiota to urolithins [Cerda *et al*., 2004, 2005; González-Barrio *et al.*, 2011], and occur in plasma as glucuronide or sulfate conjugates at nM concentrations [Larrosa *et al*., 2010a]. Urolithins exhibit documented anti-inflammatory [Giménez-Bastida *et al*., 2012; Larrosa *et al*., 2010b], chemopreventive [Da Silva Pinto *et al*., 2008] and anti-carcinogenic properties [Seeram *et al*., 2007].

Seasonability of harvest and short shelf life of strawberry fruits make them common raw material for the production of frozen products, concentrated juices, beverages, nectars and jams [Oszmiański *et al*., 2009]. Industrial processing of fruits results in vast quantities of by-products that may still contain valuable substances and therefore increase the bio-potential of the processed products [Balasundram *et al.*, 2006]. Industrial strawberry press cake remaining after juice production constitutes *ca.* 4% of the weight of processed fresh fruits [Aaby *et al*., 2005; Sójka *et al*., 2013]. After drying, strawberry pomace, and especially the part rich in pulp, contains *ca.* 60% of dietary fibre, 20% of protein, 5.4% of poly-

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phenols (mainly flavanols and ellagitannins) as well as from 3 to 8% of sand [Sójka *et al*., 2013].

A high content of sand curbs the use of strawberry pomace in food fortification with polyphenolic compounds. These compounds may be recovered through the isolation of biologically-active extracts from pomace. Such a concept fits within the strategy of more complete exploitation of the biological potential of fruits through the production of polyphenolic and fibre-polyphenolic extracts from waste materials [Schieber *et al*., 2001].

The simplest, though the least effective extraction method, is water extraction which results in the production of a polyphenolic-fibre preparation. More effective extraction is that with the use of organic solvents which enables minimising the content of carbohydrates in the resultant extract. Due to differences in the chemical composition of fruit pomace and diverse efficiency of various solvents, the extracts may differ in composition and physiological properties [Kosmala *et al*., 2014]. Results of earlier studies by Aprikian *et al*. [2003] demonstrate that easily-fermentable dietary fibre may play protective functions, compared to effects of polyphenols, on the functioning of gut ecosystem.

The aim of this study was to determine effectiveness of two methods of polyphenols extraction from strawberries: enzymatically-assisted water extraction and extraction with an aqueous solution of acetone, and then to evaluate physiological effects of the resultant preparations in the gastrointestinal tract and metabolism of rats.

MATERIAL AND METHODS

Production of ellagitannin preparations from strawberry

Enzymatically-assisted water extraction (EF preparation)

Strawberry press cake (750 kg) was taken from the concentrated juice production line of the Alpex Company (Łęczeszyce, Poland) and dried in an industrial vacuum dryer (Polfarmex Company, Kutno, Poland) at a temperature of 70±2°C. After drying, the pomace (400 kg) was separated into a seed fraction (diameter 0.5–1.0 mm) and a seedless fraction (diameter 1–3 mm) by means of proper sieve equipment. Next, the 1–3 fraction was subjected to enzyme-assisted extraction as follows: 225 kg of the 1–3 fraction of the pomace was added to 1500 L of water and 0.025 L of cellulase – pectinase enzyme (Viscozyme L) and kept at 65±1°C for 90 min. The extract was then centrifuged on an industrial centrifuge. Two further extractions were carried out as previously but each using 750 L of water. The water extracts obtained were then pooled, filtered and concentrated to a soluble solids content of *ca.* 20°Brix (digital refractometer PR-32α; Atago, Tokyo, Japan). Afterwards, part of water was freeze-dried in a TG 5 lyophilizer (VEb Hochvakuum Dresden, Germany), which enabled obtaining a raw ellagitannin-fibre preparation (EF).

Extraction with aqueous solution of acetone and selective concentration onto adsorbent (EP preparation)

A water-acetate concentrate of strawberry ellagitannins was produced in a laboratory scale using 1.5 kg of dried industrial, seedless strawberry pomace, which was not subjected to water enzyme-assisted extraction, and 60% aqueous solution of acetone (5 L per 1 kg of pomace). The extraction was run in two stages, at 25°C, for 18 h. Next, after partial removal of the solvent *via* distillation, the resultant *ca*. 5% solutions were subjected to adsorption using 500 g of Amberlite XAD 16 adsorbent in a column with a diameter of Φ 40 mm and height of 1000 mm. Then, after water elution of saccharide impurities, polyphenols were desorbed with 10, 40 and 60% ethanolic solutions applied successively in the volumes of 0.5, 1.0 and 2.0 L per 1 kg of adsorbent. Solutions containing desorbed polyphenols from acetone extraction were collected in fractions, of which only the eluates rich in ellagitannins were concentrated to *ca.* 15% of dry matter and freeze-dried.

Chemical analyses of polyphenolic extracts

Determination of the chemical composition of extracts

The chemical composition of preparations was determined using the following AOAC methods [2005]: dry matter and ash content – 940.26, protein content – 920.152; crude fat – 930.09, total dietary fibre (TDF) – 985.29, and insoluble dietary fibre (IDF) – 991.42. Soluble dietary fibre (SDF) was calculated as follows: SDF = TDF – IDF.

Determination of polyphenols content, including ellagitannins and free ellagic acid

Contents of free ellagic acid (EA), sum of ellagitannins and other polyphenols were determined with the HPLC method in solutions of ellagitannin preparations with the concentration of 1 to 5 mg/mL of solvent. To this end, respectively 25 mg of EF extract and 5 mg of EP extract were weighed into 10-mL flasks, then 8 mL of 70% glycerol solution was added to the flask, and the mixture was mixed in an ultrasound bath to complete dissolution. The dissolved mixture was filled up to the volume of 10 mL with the same solvent. When needed, the solution was filtrated or clarified *via* centrifugation. Thus prepared solution was determined for ellagic acid and polyphenols with the below-described HPLC method, whereas the sum of ellagitannins was determined based on the quantity of ester-bound ellagic acid which was calculated from the difference between total and free ellagic acid. To determine the content of total ellagic acid, 0.5 mL of clarified solution of ellagitannins in 70% glycerol and 75 μL of concentrated TFA were poured into a 2-mL flask. Next, the mixture was thoroughly mixed and kept at a temp. 95±1°C for 8 h. After completed hydrolysis, the sample was quantitatively transferred into a 5-mL measuring flask and filled up with methanol, thus obtaining a stock solution of ET which after filtrating through teflon syringe filters with pore size of 0.45 μm (Millipore, Bedford, MA, USA), was analysed in the HPLC system. The content of released EA, in mg/100 g material, was computed from the difference between determined total and free EA. The total content of ET, expressed per monomer of α-1-O-galloylo-2,3:4,6-bis-HHDP-D-glucose, was computed by multiplying content of released EA by conversion factor of 1.55. This factor takes account of the molar content of el-

lagic acid in α-1-O-galloylo-2,3:4,6-bis-HHDP-D-glucose which is a monomeric unit of agrimonine [Klimczak *et al*., 2011]. The above analytical procedure was repeated twice for each analysed sample of the material.

Chromatographic analysis was conducted with a chromatograph by KNAUER Smartline company (Berlin, Germany) equipped in degasser, two pumps, mixer, autosampler, thermostat and PDA detector. Separation was carried out onto a Phenomenex Gemini 5u C18 110A 250×4.60 mm column; 5 μm (Phenomenex, Torrance, CA, USA). The column was thermostated at 35°C. Phase A was 0.05% phosphoric acid in water, and phase B was 0.05% phosphoric acid in acetonitrile. The flow rate of the liquid phase was 1.25 mL/min. Separation was conducted in the following gradient system: 0–5 min 4% B; 5–12.5 min 4–15% B; 12.5–42.5 min 15–40% B; 42.5–51.8 min 40–50% B; 51.8–53.4 min 50–55% B; and 53.4––55 min 4% B. Volume of injected sample was: 20 μL. Conditions of detection were as follows: 280 nm (*p*-coumaric acid, kaempferol-3-O-β-D-(6″-E-p-coumaroyl)-glucopyranoside), 360 nm (ellagic acid, quercetin and kaempferol glycosides, quercetin, kaempferol), and 520 nm (anthocyanins). Data were registered using ClarityChrom software for data collection and processing (Knauer, Berlin, Germany).

Polyphenols were identified using the following standards: ellagic acid, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin, kaempferol, pelargonidin-3-O-glucoside, kaempferol-3-O-β-D-(6″-E-p-coumaroyl)-glucopyranoside (KpCG, tiliroside) Extrasynthese (Genay, France), and *p*-coumaric acid (Sigma-Aldrich, Steinheim, Germany).

Analysis of proanthocyanidins and free catechins

Proanthocyanidins degradation method in an acidic environment with an overdose of phloroglucinol was used. The method was described by Kennedy & Jones [2001]. About 20 mg of a sample was weighed to a 2 mL Eppendorf tube, and then, 800 μL of a methanol solution containing phloroglucinol (75 g/L) and ascorbic acid (15 g/L) was added to the sample. Phloroglucinolysis was started by adding 400 μL of 0.2 mol/L hydrochloric acid in methanol. The incubation was carried out for 30 min at the temperature of 50°C. Next, the samples were instantly cooled down in an ice bath, and the reaction was stopped by adding 600 μL of a 40 mmol/L sodium acetate solution. The samples were centrifuged for 5 min at 3600×*g* and then diluted with a 40 mmol/L sodium acetate solution. Products of acidic degradation of proanthocyanidins were separated with the Knauer Smartline chromatograph (Berlin, Germany) equipped with a UV–Vis P2800 detector (Knauer, Berlin, Germany) and a fluorescent detector (FD) RF-10AXL (Schimadzu, Tokyo, Japan). The separation was conducted on Gemini 5u C18 110A 250 mm 9 4.6 mm, 5 μm column with gradient elution with 2.5% water solution (v/v) of acetic acid (phase A) and 80 % (v/v) acetonitrile in water (phase B). The following gradient was used: 0–10 min, 4–7% B; 10–27 min, 7–30% B; 27–29 min, 30–70% B; 29–34 min, 70% B; 34–35 min, 70–4% B; and 35–40 min, 4% B. The flow rate was 1 mL/min, the temperature was 25°C and the volume of injection was 20 μL. The identification of components was conducted on the basis of comparing the retention times and UV–Vis spectra of standards of (-)-epicatechin, (+)-catechin, (-)-epigallocatechin, adducts: (-)-epigallocatechin-phloroglucinol, (-)-epicatechin-phloroglucinol and (+)-catechin-phloroglucinol. Quantitative analyses of released flavonols, *i.e.* (+)-catechin and (-)-epicatechin, were conducted on the basis of chromatograms recorded with the FD detector set at 278 nm excitation wavelength and 360 nm emission wavelength. Phloroglucinol adducts were determined on the basis of chromatograms registered with the PDA detector set at 280 nm wavelength.

In vivo experiments

Animals and diet

The experiment was conducted on 24 male Wistar rats aged 35 days and weighing 99.2 g (SEM 0.586), as approved by the Local Ethical Commission for Experiments with Animals in Olsztyn. The animals were maintained under standard conditions: temperature of 21–22°C and relative air humidity of 50–70%, intensive ventilation of rooms (15×/h), and 12-h lighting. Individual body weights and food intakes were recorded. The experiment, carried out in 3 experimental groups 8 male rats each, lasted 4 weeks (from week 4 to week 8 of animals life).

The experimental cholesterol-containing diets were composed according to the AIN-G93G procedure [Reeves, 1997]. The ellagitannin-fibre preparations (EF or EP) were introduced to the diet instead of the respective quantity of maize starch. The analysed preparations were used in the diet in the same dose, achieving 0.03% of the sum of free ellagic acid and ellagitannins expressed per galloyl-di-HHDP-glucose. The composition of experimental diets was presented in Table 1. Fructooligosaccharides (FOS) were applied in all diets as dietary fibre in order to attenuate the potential negative effect of polyphenols on fermentative functions of the gastrointestinal tract [Juśkiewicz *et al*., 2011]. The commercial FOS preparation (Actilight 950P) was kindly provided by Beghin Meiji (Chevrières, France). The content of oligo-, di-, and mono-saccharides in the preparation was confirmed using an HPLC system (Knauer, Berlin, Germany) equipped with a EuroChrom 2000 data control system, RI K-2301 Knauer detector (Berlin, Germany) and a Shodex NH2P (250 × 4 mm) column filled with aminopropyl polymer using a mobile phase acetonitrile-water mixture (67–33%, v/v) with a flow rate of 0.8 mL/min at 20°C. The analysed composition of the FOS preparation was as follows: fructose, 0.3%; glucose, 0.4%; sucrose, 2.3%; kestose, 36%; nystose, 49%; and fructosylnystose, 12%.

Experimental procedures

Individual feed consumption and body weight gains of rats were determined. Rats were kept in balance cages, which enabled periodical collection of urine and assessment of the diuretic effect of the analysed preparations. After the 4-wk experiment, the rats were starved overnight, weighed and anesthetised with sodium pentobarbitone. Blood samples were taken from the caudal vein. Serum was prepared by centrifugation at 1500×*g* for 15 min at 4°C and stored at -40°C until analysed. The concentration of triacylglycerol (TG), to-

TABLE 1. Composition of experimental diets (%).

	Group[1]		
	C	EF	EP
Casein	14.8	14.8	14.8
DL-Methionine	0.2	0.2	0.2
FOS[2]	5.0	5.0	5.0
Soybean oil	8.0	8.0	8.0
Cholesterol	0.5	0.5	0.5
Mineral mix	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2
Maize starch	66.8	66.4	66.7
Polyphenolic-fibre preparation	–	0.389	–
Polyphenolic preparation	–	–	0.051
Ellagitannins content in a diet	–	0.029	0.030
Polyphenols content in a diet (%)	–	0.030	0.040

[1]C – control diet without supplementation, EF – diet with ellagitannins and dietary fibre from strawberry (water extraction), and EP – diet with ellagitannins and proanthocyanidins from strawberry (acetone extraction); [2]FOS – fructooligosaccharides (Actilight 950P, Beghin Meiji, Chevrières, France).

TABLE 2. Yield of dry matter and ellagitannins extraction from strawberry pomace with two extraction methods.

	Extraction method[1]	
	water	acetone
Effectiveness of dry matter extraction from pomace		
– dry matter extracted from 1 kg of pomace (g)	67.0	22.0
– extraction yield[2] (%)	7.0	2.3
Effectiveness of ellagitannins extraction from pomace[3]		
– ellagitannins extracted from 1 kg of pomace (g)	4.7	13.0
– extraction yield[2] (%)	31.3	86.7
Ellagitannins content in dry matter of produced preparation	7.1	58.9

[1]Enzyme-assisted water extraction and acetone extraction followed by selective desorption of ellagitannins from adsorbent; [2]Extracted quantity respective to dry matter content in 1 kg of pomace; [3]Extracted quantity respective to 15 g of ellagitannins in pomace.

tal cholesterol (TC), HDL fraction of cholesterol (HDL-C) and glucose in the serum were determined with commercial diagnostic kits Alpha Diagnostics (Warsaw, Poland) and Pointe Scientific (Warsaw, Poland). HDL-cholesterol was measured after selective precipitation of low and very low-density serum lipoproteins with polypropylene glycol (PEG-600) and further removal by centrifugation (1500×*g* for 15 min at 4°C). The atherogenic index (AI) of a diet was calculated for each animal according to the formula AI = log (TG/HDL). The rats were then killed by cervical dislocation. After laparotomy, the selected tissues (liver, kidneys, heart) as well as segments of the gastrointestinal tract (small intestine, caecum and colon, including digesta) were taken from each rat. As soon as possible after euthanasia (*ca.* 10 min.), small intestine, caecal, and colonic pH was directly measured using a microelectrode and pH/ION meter (model 301, Hanna Instruments, Vila do Conde, Portugal). The small intestine was weighed with the contents. Samples of caecal and colonic contents were immediately transferred to microfuge tubes, which were stored at –70°C. The selected parts of the gastrointestinal tract (caecum and colon) were flushed clean with ice-cold saline, and blotted on filter paper, and finally weighed. Dry matter of small intestinal and caecal digesta was determined at 105°C for 4 h. In fresh caecal digesta samples, ammonia was extracted and trapped in a solution of boric acid in Conway dishes and was determined as reported elsewhere [Jurgoński *et al*., 2008a]. The activity of bacterial enzymes (α- and β-glucosidase, α- and β-galactosidase and β-glucuronidase) released into the caecal environment was measured by the rate of *p*- or *o*-nitrophenol release from their nitrophenylglucosides, according to the method described elsewhere [Wronkowska *et al*., 2011]. The enzymatic activity was expressed as μmol product formed per an hour per g of digesta. Caecal digesta samples were subjected to SCFA analysis, using GC (Shimadzu GC-2010, Kyoto, Japan). The samples (0.2 g) were mixed with 0.2 mL of formic acid, diluted with deionised water and centrifuged at 7211×*g* for 10 min. The supernatant was loaded onto a capillary column (SGE BP21, 30 m × 0.53 mm) using an on-column injector. The initial oven temperature was 85°C and was raised to 180°C by 8°C/min and held for 3 min. The temperatures of flame ionization detector and the injection port were 180 and 85°C, respectively. The sample volume for GC analysis was 1 μL.

Lipid peroxidation products in the tissue of the internal organs (heart, kidneys, liver) were assessed by reaction with thiobarbituric acid as TBARS, according to the method of Uchiyama & Michara [1978]. TBARS were determined spectrophotometrically using malondialdehyde to establish the standard curve. The results were expressed as nanomoles of TBARS per gram of tissue or mL of serum.

Statistical analysis

All results obtained were worked out statistically using a one-way analysis of variance and the Duncan's multiple range tests at a significance level of $P \leq 0.05$ using STATISTICA 6.0 (StatSoft Corp., Cracow, Poland) software.

RESULTS

Chemical composition of polyphenolic preparations

Strawberry pomace used in the study contained 95.2% of dry matter and 1.5% of ellagitannins on dry matter basis. Water extraction enabled eluting 7% of dry matter of strawberry pomace and recovering 31.3% of ellagitannins contained in the pomace (Table 2). In turn, extraction with water solution of acetone and selective desorption of ellagitannins from adsorbent allowed achieving 2.3% of dry matter of strawberry pomace and 86.7% of the initial ellagitannins content.

In the produced EF preparation, the content of ellagitannins reached 7.8% of polyphenols. In addition, this preparation contained 33% of soluble dietary fibre, over 30% of other carbohydrates and below 10% of such components

TABLE 3. Chemical composition of ellagitannin preparations (%).

	Composition of preparation		Composition of polyphenolic fraction	
	EF	EP	EF	EP
Basic composition				
Dry matter	95.6	93.6	-	-
Protein	8.0	0.5	-	-
Fat	0.1	0.0	-	-
Ash	7.5	0.2	-	-
Soluble dietary fibre (SDF)	33.3	0.0	-	-
Other carbohydrates[1]	38.9	14.4	-	-
Sum of polyphenols	7.8	78.5	100	100
Free ellagic acid	0.4	0.6	5.1	0.8
Ellagitannins[2]	7.1	58.9	91.0	75.0
P-coumaric acid and derivatives	0.0	0.2	0.0	0.3
Flavonoids[3]	0.1	1.0	1.3	1.3
Proanthocyanidins	0.2	16.9	2.6	21.5

[1]Carbohydrates, including polysaccharides of cell walls (without polyphenols) extracted from fruits and calculated from the difference between dry matter and determined components, including SDF; [2]Ellagitannins expressed as galloyl-bis-HHDF-glucose equivalent; [3]Flavonoids = quercetin and kaempferol with their glycosides and anthocyanins.

as: crude protein, ether extract and ash (Table 3). In the EP preparation produced *via* aqueous acetone extraction followed by selective adsorption and desorption onto Amberlite XAD 16, the content of ellagitannins reached 58.9%. The second fraction of polyphenols in terms of quantity were proanthocyanidins (16.9%). The EP preparation did not contain SDF, in turn it contained a small quantity of other carbohydrates. In both preparations, ellagitannins were the main fraction of polyphenolic compounds and constituted 91% and 75% of the sum of polyphenols in EF and EP preparation, respectively. Compared to EF preparation, the EP preparation was characterised by a significantly higher content of proanthocyanidins (21.5 *vs.* 2.6% of total polyphenols) and by a lower content of ellagic acid (0.8 *vs.* 5.1% of total polyphenols).

Diet intake and parameters of rats growth and development

The use of ellagitannin preparations in experimental diets had no effect on diet intake, body weight gains and mass of internal organs (heart, liver and kidneys) of the rats (Table 4). Values of daily diet intake (14.2 g on average) and strawberry ellagitannins content in the diet (0.03%) demonstrate that the daily intake of ellagitannins reached 0.024 g/kg body weight (BW). When converted in metabolic unit of body size of rat (0.265 $kg^{0.75}$) and adult man (24.2 $kg^{0.75}$), it reached 0.015 $g/kg^{0.75}$ and 0.36 g/day, respectively.

The EF preparation had no effect on small intestine bulking, whereas EP tended to decrease (P=0.087 *vs*. EF) the mass of small intestine with digesta. The EP preparation statistically significantly (P=0.014 *vs.* C) decreased the mass of digesta in the caecum. Both preparations significantly reduced the mass of caecum wall compared to the control group (P<0.05 *vs.* C). The ellagitannin preparations used in the diet had no impact on dry matter content in caecum digesta. They were also characterised by lower concentration of ammonia, compared to the control group. Statistical tendency was noted for a higher pH value in caecal digesta of rats fed the EP diet (P=0.099 *vs.* EF).

Fermentation processes in the caecum of rats

The supplementation of diets with EF or EP preparations did not affect the activities of bacterial glycolytic enzymes released into the caecal environment, *i.e.* α- and β-glucosidase as well as α- and β-galactosidase (Table 5). Dietary addition of preparation EP tended to decrease the activity of bacterial β-glucuronidase in the caecal digesta (P=0.060 *vs.* C). Compared to group C, in group EF there was a small decrease whereas in group EP there was a statistically significant decrease in the concentration of acetic acid and in SCFA sum in caecal digesta (P=0.026 and P=0.030, respectively). In the rats fed the diet with EF preparation analyses showed the highest concentration of butyric acid in acetic digesta (P<0.05 *vs.* EP). The analysis of SCFA C2:C3:C4 profile points to a higher ratio of propionic acid under the influence of diets with EP addition (P<0.05 *vs.* treatments C and EF).

Biochemical blood markers and TBARS value in selected organs

The study demonstrated varied effects of ellagitannin preparations on levels of the analysed biochemical blood markers and concentration of thiobarbituric acid-reactive substances (TBARS) in selected organs (Table 6). Considering glucose blood level, the experimental groups could be ordered as follows: C > EF > EP (P=0.001). The serum level of triacylglycerols was significantly (P=0.001) lower in EP rats, compared to animals from groups C and EF. No differences were noted, in turn, in concentrations of TC and HDL-C in serum, whereas the content of HDL fraction in total cholesterol was significantly lower in EF group than in the other analysed groups (P=0.035). In contrast, a significantly lower atherogenic index of diet (log (TG/HDL-C)) was determined in group EP (P=0.001 *vs*. other groups).

TABLE 4. Diet intake, growth rate and intestinal indices of rats.

	Experimental group[1]			SEM	*P* value
	C	EF	EP		
Diet intake (g)	392	395	403	4.447	0.427
Body weight (g)					
initial	99.3	99.4	98.9	0.586	0.792
final	241	242	242	2.495	0.793
Mass of organs (g/100 g BW)					
Heart	0.274	0.274	0.280	0.004	0.565
Liver	4.43	4.28	4.40	0.082	0.505
Kidneys	0.683	0.648	0.651	0.008	0.101
Epididymal fat	1.25	1.20	1.27	0.072	0.739
Full small intestine (g/100 g BW)	2.80	2.98	2.66	0.072	0.087
Caecum					
tissue (g/100 g BW)	0.881[a]	0.713[b]	0.640[b]	0.037	0.009
digesta (g/100 g BW)	2.77[a]	2.45[ab]	1.92[b]	0.139	0.014
ammonia (mg/g digesta)	0.312[a]	0.262[b]	0.275[b]	0.007	0.032
dry matter (%)	17.6	17.9	17.8	0.158	0.489
pH of digesta	6.34	6.27	6.56	0.068	0.099

[1]C – control diet without supplementation, EF – diet with ellagitannins and dietary fibre from strawberry (water extraction), and EP – diet with ellagitannins and proanthocyanidins from strawberry (acetone extraction); [a,b] Mean values within a row with unlike superscript letters were shown to be significantly different (P<0.05).

TABLE 5. Activity of microbiota enzymes and concentration of short-chain fatty acids in caecal digesta.

	Experimental group[1]			SEM	*P* value
	C	EF	EP		
Enzyme activity (μmol/h/g digesta)					
α-glucosidase	18.7	20.5	20.8	1.001	0.435
β-glucosidase	1.31	1.40	1.83	0.132	0.133
α-galactosidase	5.29	4.16	5.43	0.400	0.231
β-galactosidase	15.0	14.9	12.5	0.891	0.277
β-glucuronidase	4.73	4.38	3.20	0.317	0.060
SCFA (μmol/g digesta)					
C2	30.5[a]	29.1[ab]	23.7[b]	1.222	0.026
C3	7.65	6.39	7.85	0.323	0.078
C4i	0.331	0.252	0.281	0.028	0.280
C4	6.99[ab]	7.66[a]	5.48[b]	0.434	0.050
C5i	0.550	0.442	0.420	0.032	0.129
C5	0.971	1.53	0.991	0.142	0.123
Total SCFA	47.0[a]	45.4[ab]	38.8[b]	1.517	0.030
SCFA profile (% of total SCFA)					
C2	64.6	63.7	61.3	0.965	0.202
C3	16.2[b]	14.2[b]	20.2[a]	0.724	0.001
C4	15.2	17.1	14.0	0.899	0.192

[1]C – control diet without supplementation, EF – diet with ellagitannins and dietary fibre from strawberry (water extraction), and EP – diet with ellagitannins and proanthocyanidins from strawberry (acetone extraction); [a,b] Mean values within a row with unlike superscript letters were shown to be significantly different (P<0.05).

The preparations of ellagitannins had no effect on the level of thiobarbituric acid-reactive substances (TBARS) in heart tissue of rats. The highest concentration of TBARS in the liver tissue ($P<0.05$ *vs.* EF) and kidneys ($P<0.05$ *vs.* EF and EP) was observed in the group C.

TABLE 6. Biochemical blood markers and content of thiobarbituric acid-reactive substances (TBARS) in selected organs of rats.

	Experimental group[1]			SEM	*P* value
	C	EF	EP		
Serum parameters					
Glucose (mmol/L)	8.61[a]	7.80[b]	6.79[c]	0.203	0.001
Triacylglycerols (TG) (mmol/L)	1.67[a]	1.56[a]	0.98[b]	0.084	0.001
Total cholesterol (TC) (mmol/L)	3.05	3.27	2.78	0.120	0.119
HDL cholesterol (mmol/L)	1.71	1.63	1.55	0.061	0.346
HDL cholesterol (% of TC)	56.0[a]	50.1[b]	55.7[a]	1.124	0.035
log(TG/HDL)	-0.004[a]	-0.018[a]	-0.219[b]	0.028	0.001
TBARS (nmol/g tissue)					
Heart	43.5	42.6	36.3	1.578	0.075
Liver	49.6[a]	41.3[b]	44.3[ab]	1.281	0.008
Kidneys	58.5[a]	53.4[b]	53.5[b]	0.968	0.020

[1]C – control diet without supplementation, EF – diet with ellagitannins and dietary fibre from strawberry (water extraction), and EP – diet with ellagitannins and proanthocyanidins from strawberry (acetone extraction); [a,b] Mean values within a row with unlike superscript letters were shown to be significantly different ($P<0.05$).

Diuretic effect

The daily volume of excreted urine was increasing along with the age of rats and depended on diet composition (data not shown). As indicated by the calculated diuretic coefficients, both preparations of ellagitannins exhibited the diuretic effect, *i.e.* were increasing the volume of excreted urine compared to urinal excretion in control rats (Table 7).

TABLE 7. Diuretic effect of dietary strawberry preparations.

Day	Diuretic effect coefficient[1]	
	EF	EP
1	1.8±0.7	2.9±2.5
2	2.6±0.8	3.8±1.7
3	1.2±0.1	1.6±0.2
4	1.7±0.2	2.1±0.7
8	1.1±0.5	2.1±1.1
14	1.4±1.4	1.8±0.2
22	1.5±0.6	2.3±0.8
Average	1.4±0.6	2.0±0.6

[1]Ratio of the volume of excreted urine in group EF and EP to the volume of urine excreted in group C (C – control diet without supplementation, EF – diet with ellagitannins and dietary fibre from strawberry (water extraction), and EP – diet with ellagitannins and proanthocyanidins from strawberry (acetone extraction).

DISCUSSION

The content of ellagitannins in pomace (1.5%) used in experimental cholesterol-containing diets was similar to values reported in literature [Sójka *et al.*, 2013]. Compared to the average content of ellagitannins in fresh fruits, estimated at 0.064% by Gasparotti *et al*. [2013], their concentration in pomace is over 20-times higher, which facilitates their extraction and makes pomace an attractive, additional source of dietary ellagitannins.

The ellagitannin preparation produced in the semi-technical scale by enzymatically-assisted water extraction contained nearly 70% of the carbohydrate fraction and 7.8% of polyphenols including mainly ellagitannins and free ellagic acid. In terms of the composition of the polyphenolic fraction, the EF preparation was similar to the water extract obtained in an earlier study by Kosmala *et al*. [2014]. In the presented experiment, the addition of enzymes in the process of water extraction increased the content of soluble dietary fibre to 33%, compared to its 22% content noted in the preparation produced through three-stage water extraction at 65–70°C [Kosmala *et al*., 2014]. This result indicates that the additional application of glycolytic enzymes in water extraction of ellagitannins, aimed at reducing energy consumption of this process, does not change the composition of the polyphenolic fraction, but only increases the content of soluble dietary fibre in the resultant product.

Many authors have proposed that the beneficial activity of polyphenolic compounds present in fruit preparations is also linked with physiological effects of dietary fibre constituting the fibre-polyphenols complexes [Larrauri *et al*., 1996; Juśkiewicz *et al*., 2011]. Some of them have even postulated the necessity of characterising parameters of the antioxidant status of the body at dietary supplementation with different dietary fibres [Larrauri *et al*., 1996]. The fibre-antioxidants complex seems to be a natural route of delivering components with antioxidative properties to colonic microbiota, thus protecting antioxidants against degradation in the stomach [Esposito *et al.*, 2005; Saura-Calixto, 2011; Vitaglione *et al*., 2008; Jurgoński *et al*., 2011]. These studies point to potential advantages of diet supplementation with natural preparations containing both functional polysaccharides and polyphenolic compounds, which enables utilizing the physiological properties of both groups of these compounds locally in the gut as well as in internal tissues. Our recent study showed that the addition of the polyphenolic fraction from chicory root to diets containing prebiotic fructans did not diminish the positive effect of inulin and oligofructose on the ecosystem of the gastrointestinal tract, and triggered positive changes

in the lipid profile of blood serum as well as deceleration of pro-oxidative processes in selected tissues [Juśkiewicz *et al*., 2011]. In the present study, all diets were supplemented with 5% FOS but the additional soluble fibre from the EF preparation might affected some analysed parameters. Indeed, some differences between physiological response to dietary treatments EF and EP, discussed below, were observed.

In the current experiment, extraction with aqueous acetone enabled producing a preparation with ellagitannin recovery effectiveness of 86% and a high content of polyphenols including proanthocyanidins (21.5% of polyphenols sum) being complementary to ellagitannins. It is also known that both condensed and hydrolysing tannins are strongly bound with saccharides of plant tissue walls [Arapitsas, 2012]. It explains why even after enzymes addition the content of proanthocyanidins in the water extract was very low (2.6% of total polyphenols).

In many earlier experiments with the use of similar or even higher concentrations of fruit polyphenols in diets, no negative effects of these compounds were demonstrated on feed intake and growth of experimental animals [Zduńczyk *et al*., 2006; Frejnagel & Juśkiewicz, 2011; Jurgoński *et al*., 2011; Kosmala *et al*., 2011, 2014]. In the presented study, doses of ellagitannins applied in a daily diet (0.015 and 0.016 g/kg $BW^{0.75}$) did not affect feed intake and body weight gains of rats. Such a result was achieved at a relatively high ellagitannin content in diets that corresponded to a daily intake of ellagitannins by adult man (0.36 and 0.39 g, respectively) and to ellagitannins content in 550 and 600 g of strawberry fruits. Results of previous investigations demonstrate that dietary polyphenols may reach any segment of the gastrointestinal tract in measurable quantities and modify the functioning of its ecosystem [Manach *et al*., 2005]. It applies to polymerised polyphenols, including ellagitannins. Only trace amounts of ellagitannin metabolites are absorbed in the stomach and small intestine [Espin *et al*., 2007], however, the majority of these substances are metabolised by the large intestinal microbiota [Mertens-Talcott *et al*., 2006; Tomas-Barberan *et al*., 2008]. The presence of polyphenols in digesta may affect the basic functions of intestine, including hydration and pH value of digesta [Zdunczyk *et al*., 2006], caecal digesta mass [Kosmala *et al*., 2014; Jurgoński *et al*., 2011] and ammonia concentration [Juśkiewicz *et al*., 2011]. In the reported experiment, both preparations were observed to similarly lower ammonia concentration in digesta and to differently affect the mass and pH value of caecal digesta, namely the ET preparation with dietary fibre had no effect on these parameters whereas the ET preparation with proanthocyanidins (with ratio of both groups of polyphenols at *ca.* 3:1) significantly decreased the mass and increased the pH value of caecal digesta, compared to the control group.

The reduced ammonia concentration in caecal digesta, determined in the reported experiment, points to favourable suppression of microbiological proteolysis and then deamination of amino acids in the caecum. This result could be found beneficial as ammonia not consumed by microbiota may cause damage to mucosa, disorders or microcirculation and even damage to epithelial cells [Hambly *et al*., 1997].

In our experiment, the addition of both ET preparations to diets did not affect the activity of microbial α- and β-glucosidase as well as α- and β-galactosidase. Results of other studies demonstrate that dietary addition of polyphenols may suppress the activity of gut microbiota [Negi & Jayaprakasha, 2001], including the activity of such bacterial enzymes like β-glucosidase and β- and α-galactosidase [Zduńczyk *et al*., 2006]. Such an effect was observed in the presented experiment for microbial β-glucuronidase, the activity of which was significantly decreased by EP addition to diet. The significant decrease of β-glucuronidase activity was also demonstrated in another study with the use of water extract of strawberry ellagitannins [Kosmala *et al*., 2014]. A research by Klewicka *et al*. [2009] showed that an increase in β-glucuronidase occurred upon increased counts of *Escherichia coli* and *Clostridium* bacteria in intestinal digesta. In this context, the determined reduction in β-glucuronidase activity may be found a symptom of beneficial changes in the intestinal ecosystem under the influence of strawberry polyphenols. Such a possibility was also demonstrated in a study where ellagitannins from pomegranate and their metabolites evoked positive changes in gut microbiota profile by reducing the count of enterobacteria and increasing the count of bacteria representing *Lactobacillus* and *Bifidobacteria* genera [Larrosa *et al*., 2010b]. In another study, Bialonska *et al*. [2009] demonstrated that ellagitannins were suppressing the growth of pathogenic bacteria from the genus *Clostridium* and *Staphyloccocus aureus*, and significantly enhanced the growth of *Bifidobacterium breve* and *Bifidobacterium infantis*.

In our experiment, preparations of ellagitannins had diverse effects on the concentration of butyric acid in caecal digesta, *i.e.* compared to the control group the ET preparation with dietary fibre increased, whereas the ET preparation with proanthocyanidins reduced the concentration of C4 acid. Such a result points to a more favourable effect of the preparation produced *via* water extraction that caused elution of ellagitannins and, even more, of carbohydrates including soluble dietary fibre. It is well known that SCFA are rapidly absorbed and metabolised by various tissues, including liver (propionate and partly acetate), muscle (acetate) and intestinal epithelium (butyrate) [Priebe *et al*., 2002]. For this reason, butyric acid is more effective in inducing the proliferation of intestinal epithelial cells than acetic acid, the main product of caecal fermentation in poultry [Williams *et al*., 2001].

Results of earlier investigations show that the presence of polyphenolic compounds in intestinal digesta may be a factor reducing postprandial glycaemia by suppressing the activity of glycolytic enzymes and/or decelerating glucose transport through walls of intestinal epithelium [Jurgoński *et al*., 2008b]. Such an effect, stronger in the case of the preparation produced *via* acetone extraction, may explain the fact of reduced glucose level in blood of rats receiving both ET preparations in diet.

Results of many studies demonstrate the positive impact of dietary polyphenols on the level and composition of blood lipids [Jenkins *et al*., 2008; Basu *et al*., 2009; Jarosławska *et al*., 2011]. In the presented experiment, such an effect was observed for ET preparation produced *via* acetone extraction, whereas the preparation produced by water extraction did not

cause the beneficial reduction of cholesterol and/or triacylglycerols level in blood. It may be speculated that the presence of anthocyanidins alone or their appropriate complex with ellagitannins in the EP preparation was the factor which reduced TG level and, thus, had a beneficial effect on the atherogenic index of diet.

In our experiment, a lower concentration of thiobarbituric acid-reactive substances was determined in the liver of rats. It indicates antioxidative properties of ellagitannin metabolites in large part metabolised in liver [Mertens-Talcott *et al*., 2006; Tomas-Barberan *et al*., 2008; González-Barrio *et al*., 2011].

It is known that urolithins, the main metabolites of ellagitannins, after participation in the metabolic process are excreted with urine and faeces [Truchado *et al*., 2012; Espin *et al*., 2007]. Seeram *et al*. [2007] demonstrated that metabolites of pomegranate ellagitannins administered to mice are concentrating in higher quantities in intestinal tissues and in prostate. Other authors [Landete, 2011; Zhang *et al*., 2011] reported on the diuretic effect of ellagitannins. In our experiment, greater urine excretion was determined in the case of rats receiving diets with ET preparations, however analyses did not show the hypertrophy of this organ. Like in the case of liver, a reduced TBARS value was also noted in kidneys, which was indicative of the antioxidant effect of ellagitannins.

SUMMARY

Results of the conducted experiment demonstrate that the more efficient acetone extraction of strawberry pomace was increasing contents of both ellagitannins and proanthocyanidins in the polyphenolic preparation from strawberry. The intake of such preparation in diet at 0.024 g/kg BW of rats, resulted in more tangible inhibiting effect on fermentation processes in the caecum and in a positive impact on lipid profile and glucose level in blood. Considering the identical content of ellagitannins in both supplemented diets, it may be speculated that the above effects were due to the presence of proanthocyanidin fraction. The positive physiological effect of ellagitannin preparation produced *via* water extraction was less explicit, however it beneficially reduced ammonia level in caecal digesta and glucose level in blood serum of rats. Study results show the feasibility of effective recovery of ellagitannins from selected fractions of strawberry pomace (over 85%) as well as the possibility of modifying fermentation processes in the gastrointestinal tract and carbohydrates metabolism by the use of a preparation composed of ellagitannins and proanthocyanidins (75% and 21% of total polyphenols, respectively) in diet.

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