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Lemon Juice Formulations Modulate *In Vitro* Digestive Recovery of Spinach Phytochemicals

Running head: *In Vitro* Digestion of Spinach Lemon Formulations

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SUMMARY

Research background. Citrus limon (L.) Burm lemon juice is rich with many important natural chemical components (flavonoids, citric acid, vitamin C) and is used in traditional medicine is well known. Formulations of lemon juice with fruit polyphenols in beverage systems has been investigated, but there is very little information about their ability to modulate polyphenol digestive behavior. The goal of this study was to determine stability and digestive availability of spinach (*Spinacia oleracea* L.) polyphenols by adding different concentrations of lemon juice (0, 2, 5, 10 and 20 %) during *in vitro* digestion.

Experimental approach. Content of polyphenols, and other spinach rich compounds including nitrate, oxalic acid, *L*-ascorbic acid in spinach formulation with various concentrations of lemon juice were measured in predigested and digested samples using *in vitro* human digestion model. Antioxidant activity and α-amylase inhibitory activity of spinach lemon juice formulation was also measured.

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Results and conclusions. Highest increases in total polyphenols, total flavonoids, total phenolic acids, oxalic acid and nitrate content were noted in predigested and almost all digested spinach samples formulated with highest concentration of lemon juice. In the same sample concentration of individual compounds significantly increased after salivary (*L*-ascorbic acid), initial (*p*-coumaric acid) and intestinal (quercetin) phase of digestion for each phase individually. High bioaccessibility of polyphenols, nitrate, oxalic and *L*-ascorbic acid for all phases of digestion were observed in almost all spinach lemon juice formulation with the exception for nitrate (gastric and intestinal phase) and oxalic acid (intestinal phase) which had moderate bioaccessibility.

Novelty and scientific contribution. For the first time stability and digestive availability of spinach polyphenols, oxalic acid, nitrate and *L*-ascorbic acid were tested with different concentrations of lemon juice. Lemon juice pH and *L*-ascorbic acid increase stability and availability of polyphenols in spinach lemon juice formulation during *in vitro* digestion. Antioxidant and α-amylase inhibitory activity increase in dose depending manner after lemon juice addition. Accordingly, spinach formulated with 20 % of lemon juice appears as the best source of dietary polyphenols with antioxidant activity, antidiabetic activity and nitrate that may be used as functional drink.

Keywords: *Spinacia oleracea* L.; polyphenols; *L*-ascorbic acid; nitrate; oxalate; *in vitro* digestion

INTRODUCTION

Spinach *(Spinacia oleracea* L.) is a functional food that is consumed in fresh (*e.g.* salads, smoothies), cooked (*e.g.* steamed, soups, in fresh pasta) and dried forms (*e.g.* smoothies) due to its diverse nutritional and chemical composition. It is a good source of minerals (magnesium, potassium and iron), vitamins (vitamin K, vitamin A, folate and vitamin C), carotenoids (β-carotene, lutein, zeaxanthine), polyphenols (flavonoids and phenolic acids) (*1-3*). So far, various functional properties of spinach leaves and its preparations (extracts, fractions) have been studied, among which are antioxidant, anti-inflammatory, anti-proliferative, anti-obesity, hypoglycemic and lipid-lowering activities (*2*). Spinach is also rich with oxalic acid and dietary nitrates (*4,5*). Nitrate rich spinach can promote nitric oxide production, enhance endothelial function and lower blood pressure acutely. These outcomes may benefit to cardiovascular health (*6*) and exercise performance (*7*). Also natural nitrite and nitrate containing dietary can reduce triglycerides in humans (*8*). According to Zhang *et al.* (*9*) nutritive value of spinach in human diet is limited to its high oxalate content. High oxalate content in diet can disturb bioavailability of some minerals by intestine and cause deficiency of calcium, iron, magnesium and copper. Also, consumption of additional oxalate can increase the risk

of kidney stones formation by inducing a significant increase in urinary oxalate excretion (*9*). Therefore, the measurement of oxalic acid content along with other bioactive compounds is of great importance when it comes to spinach preparations. *Citrus limon* (L.) Burm lemon juice is also rich with many important natural chemical components (flavonoids, citric acid, vitamin C, and minerals *e.g.* calcium and phosphorus) and its use in traditional medicine is well known for scurvy, high blood pressure treatment, common cold treatment, and irregular menstruation (*10*). Antioxidant activity of flavonoids and vitamins C are the most important factors for its health promoting properties (*11*) contributing to reducing the symptoms of inflammation and excessive formation of reactive oxygen species and thus reducing the risk of developing cardiovascular disease, diabetes, obesity and cancer (*10,12*). Lemon juice is increasingly being utilized in fruit and vegetable drinks and in fresh brewed tea products to improve organoleptic quality and nutritive value of drink (*13,14*). Although lemon juice formulations with fruit (*11,13,15*) polyphenols in beverage systems has already been investigated, knowledge about their ability to modulate polyphenol digestive behavior is scarce. Only Green *et al.* (*14*) studied the effect of citrus juices (grapefruit, lemon, lime, and orange) on *in vitro* digestive recovery of green tea polyphenols (catechins) and reported highest catechin recoveries for lemon juice, followed by orange, lime and grapefruit juice. Digestible vitamin C and D may have positive effect on polyphenol (flavonoids: catechins and anthocyanins) content and bioavailability (*16*). The stability of the polyphenols may also be affected by the pH of the solution (*17*). The pH values and content of vitamins and polyphenols of different citrus juices depend on the variety, condition of growing, harvesting time, storage, and juice processing (*18-21*). Of the citrus most used in the preparation of formulations with fruits and vegetables, lemon has the lowest pH, while lime, grapefruit, and orange all have higher pH (*22*). Orange has the highest content of vitamin C, lemon and grapefruit follow with the similar amount, while lime contains the least of vitamin C (*14*). Lemons and oranges have similar total polyphenol content while lime and grapefruit have similar lower values (*23*).

The goal of this study was to determine stability and digestive availability of spinach polyphenols in spinach formulation with various concentrations of lemon juice (0, 2, 5, 10 and 20 %) using *in vitro* digestion model. We used dry fine spinach powder in sample preparation to potentially obtain higher values of bioactive compounds. Fine spinach powder can expose significantly higher water-binding characteristic (*3*) and therefore can have positive impact on bioactive compounds extraction (*24*). Spectrophotometric (total phenols; TP, total flavonoids; TF, total phenolic acid; TPA) and HPLC (High Performance Liquid Chromatography: individual phenolic compounds: *p*-coumaric acid; *p*-CA, ferulic acid; FA and quercetin; Q) methods were used to determine concentration of polyphenols in predigested and digested spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

and in pure lemon juice. Concentration of vitamin C (*L*-ascorbic acid, *L*-AA) as the most abundant vitamin in lemon juice was measured with HPLC in predigested (0, 2, 5, 10 and 20 %) and digested (0 and 20 %) spinach lemon juice formulations and in pure lemon juice. Spectrophotometric determination of health promoting dietary nitrate and health potentially dangerous oxalic acid (OA) was also measured in all spinach samples and in lemon juice. Determination and comparison of antioxidant activity of all predigested and digested spinach lemon juice formulation and lemon juice was performed with DPPH (1,1-diphenyl-2-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) methods. α-amylase inhibitory activity was performed to test predigested spinach formulation and lemon juice for the potential antidiabetic activity. pH of lemon juice and all predigested and digested spinach lemon juice formulation was also tested.

MATERIALS AND METHODS

Materials and preparation of spinach lemon juice formulations

Enzymes (α-amylase, porcine pepsin, lipase, pancreatin) and bile utilized for *in vitro* digestion and antidiabetic activity (α-amylase) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,5-dinitrosalicylic acid, salicylic acid (SA), 2, 2′-diphenyl-2-picrylhydrazyl hydrate (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), *L*-ascorbic acid (*L*-AA) and all phenol standards: caffeic acid (CA), gallic acid (GA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and quercetin (Q) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Acros Organics (New Jersey, USA). Folin–Ciocalteu reagent (FC) and all other chemicals were purchased from Kemika (Zagreb, Croatia). Deionized water was used for all experiments and the solvents and chemicals used were of analytical grade or HPLC grade.

Spinach BIO powder (brand: Chefica, certifications: HR-EKO-05, origin: Germany) were perched in local health food store Chefica (Lanište 15c, 10000 Zagreb, Croatia). Spinach powder (1 g) was overflowed with 50 mL of deionized boiling water (100 °C) and cooked on a laboratory heater TK 23 (TechnoKartell, Noviglio, Milan, Italy) for 40 minutes to obtain a concentrated extract of 100 mg/mL. The concentrated extract was filtered through filter paper (Whatman Grade 595). Lemons were bought in local store Konzum (sort: Primofiori, net quantity: 500 g, country of origin: Spain). Lemon juice were prepared from 500 g of squeezed lemons. Spinach lemon juice formulations were prepared by adding filtered (Whatman Grade 595) lemon juice at 0, 2, 5, 10 and 20 % (*V/V*) in concentrated extract. Spinach extracts were cooled down on 25 °C under running water flow before adding the lemon juice. Immediately after preparation of spinach lemon formulations, *in vitro* digestion of samples was performed.

Model of human in vitro digestion

In vitro digestion model was performed according to Šola *et al.* (*25)* with slight modification. First, spinach lemon formulations (0.3 mL) was mixed with 0.3 mL of phosphate buffer (20 mM, pH=7). Modification was made by adding salivary phase of digestion and initialized by 10 µL of amylase (0.48 mg/mL in 20 mM phosphate buffer pH=7) and incubated for 5 minutes at 37 °C in a shaking water bath SW22 (Julabo, Seelbach, Germany) at 150 rpm. Volume of 0.4 mL of porcine pepsin solution (3 mg/mL in 0.1 M HCl) was added and acidified with HCl (0.5 M, pH=2) for simulating the stomach digestion and incubated in a shaking water bath for 1 h at 37 °C at 150 rpm. Sodium bicarbonate (1 M) was added to adjust pH to 5.3 and mimicked upper intestinal phase of digestion. After pH adjustment volume of 0.9 mL of pancreatic juices (2.4 mg bile acids/mL, 0.2 mg porcine lipase/mL, 0.4 mg pancreatin/mL in 20 mM phosphate buffer pH=7) was added. All intestinal phase sample was brought to 2 mL with phosphate buffer (20 mM, pH=7). The final pH was adjusted additionally to 7 with 1 M NaOH and then incubated for 2 h at 37 °C in a shaking water bath at 150 rpm. The final volume of each sample, both before and after digestion, was brought to 2 mL with phosphate buffer (20 mM, pH=7). Samples were centrifuged (Hettichmikro 220R, Andreas Hettich GmbH & Co. Tuttlingen, Germany) after *in vitro* digestion at 11 000 rpm for 10 minutes at 4 °C and supernatants were stored at −20 °C until spectrophotometric and HPLC analyses.

Spectrophotometric phytochemical analysis

TP and TF were determined according to Zhishen *et al.* (*26*). For TP and TF absorbance was measured at 740 and 405 and expressed as gallic acid equivalents (GAE) and quercetin equivalents (QE), respectively. For TP determination 2 µL of tested solution was diluted with 158 µL of deionized water and then 10 µL of Foline-Ciocalteau reagent was added. Afterwards, 30 µL Na_2CO_3 (1.88 M) was added, and the mixture was incubated for 30 min at 45 °C. The content of TF was determined with $AlCl_3$. First tested sample (20 µL) was diluted within 80 µL of dH_2O, then volume of 6 µL $NaNO_2$ 5 % (*m/V*) was added. After 5 min incubation, volume of 6 µL $AlCl_3$ 10 % (*m/V*) was added and mixture was incubated at room temperature for additional 6 min. Afterwards, 40 µL NaOH (1 M) and distilled water were added to final volume of 200 µL. TPA were determined according to European Pharmacopoeia (*27*) at 492 nm and expressed as caffeic acid equivalents (CAE). Volume 40 µL of tested solution was mixed with 80 µL HCl (0.5 M). An 80 µL of freshly prepared reagent (1 g $NaNO_2$ and 1,17 g Na_2MoO_4 x $2H_2O$ in 10 mL deionized water) was added in this solution. OA content were determined with $KMnO_4$ according to the method described by Naik (*28*) at 520 nm and expressed as oxalic acid equivalents (OAE). Volume of 25 µL was mixed with 125 µL of 2 N H_2SO_4 and 50 µL of 0.003 M $KMnO_4$ and incubated for 10 minutes. The nitrate content was determined using salicylic

acid according to Cataldo *et al.* (*29*) at 405 nm and expressed as KNO_3 equivalents (KNO_3E). Volume of 50 µL of tested solution was mixed with 160 µL of salicylic acid solution 5 % (*m/V*) in H_2SO_4. After 20 min of incubation at room temperature, 950 µL of 2 N NaOH was added.

Reversed-phase high performance liquid chromatography analysis

Before HPLC analysis samples were hydrolyzed with 1.2 M HCl for 2 h at 80 °C and 300 rpm. Qualitative and quantitative RP-HPLC analysis was performed using the Agilent 1100 Series system equipped with a quaternary pump, multiwave UV/Vis detector, autosampler, fraction collector, Zorbax SB C-18 analytical guard column (12.5 x 4.6 mm, 5 µm particle size) and Poroshell 120 SB-C18 column (75 x 4.6 mm, 2.7 µm particle size) (Agilent Technologies, Waldbronn, Germany). The solvents used were: (A) 0.2 % (*V/V*) glacial acetic acid, (B) 80 % methanol and 0.2 % glacial acetic acid. Gradient profile was (A/B): 100/0 at 0 min, 20/80 at 42 min, 0/100 at 43 min, 0/100 at 45 min, 100/0 at 45.1 min, 100/0 at 48 min as in Šola *et al*. (*30*). Injection volume was 25 µL, the constant flow rate 1.0 mL/min, and the column temperature was set at 30 °C. For quantification, the multiwave UV/Vis detector was set at 254 nm for *L*-AA, 310 nm for *p*-CA and FA, and 360 nm for Q determination. Phenolic compounds and *L*-AA were characterized according to their retention times and UV spectra compared with commercial standards. For the quantitative analyses, calibration curves were obtained by injection of 5 known concentrations (in the range 1-250 µg/mL) of the mixed standard solution in triplicate. The results were expressed as mg/kg dm (dry mass).

Antioxidant activity assays

DPPH radical scavenging and ferric reducing/antioxidant power (FRAP) assay was performed as reported by Šola *et al.* (*30*) adapted to small volumes. The results are expressed in percentage of inhibition of DPPH and in µM Trolox equivalents (TE) for DPPH method and in percentage of reduction of Fe^{3+} - TPTZ and as µM Fe^{2+} for FRAP method. Briefly for DPPH method, 10 µL of tested solution was added to 190 µL of freshly prepared ethanolic DPPH solution (0.1 mM) and incubated in the dark for 30 min at room temperature. The decrease in absorbance was measured at 520 nm. A calibration curve was constructed for Trolox and expressed as Trolox equivalents (TE). DPPH radical inhibition (%) was calculated as follows:

$$\text{DPPH inhibition} = ((A_{control} - A_{sample})/A_{control}) \cdot 100 \quad /1/$$

where $A_{control}$ was the absorbance of the control (blank, without tested solution) and A_{sample} was the absorbance in the presence of the tested solution. Trolox was used as a positive control.

For FRAP method, tested solution (10 µL) was mixed with 190 µL of freshly prepared FRAP reagent (25 mL of 0.3M CH_3COONa x $3H_2O$ pH=3.6, 2.5 mL of 10 mM Tripyridil-s-triazine=TPTZ

solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3$ x 6 H_2O) and the absorbance was read at 595 nm after 4 min reaction time. A calibration curve was constructed for $FeSO_4$ x 7H_2O and the results were expressed as μM Fe^{2+} and in % of reduction of Fe^{3+} - TPTZ reduction. FRAP reduction (%) was calculated as follows:

$$\text{FRAP reduction}=((A_{sample}-A_{control})/A_{sample})\cdot 100 \quad /2/$$

where $A_{control}$ was the absorbance of the control (blank, without tested solution) and A_{sample} was the absorbance in the presence of the tested solution. Trolox was used as a positive control for both methods when the results were presented in percentage.

α-amylase inhibitory activity assay

Antidiabetic properties of predigested spinach lemon juice formulations and lemon juice through inhibition of α-amylase were tested using the pre-incubation method as described by Šola *et al.* (*25*) and expressed in percentage of inhibition. Predigested spinach lemon juice formulations and lemon juice (20 μL) was mixed with 20 μL α-amylase from human saliva (5 unit/mL solution in ice-cold distilled water) and 40 μL of 20 mM phosphate buffered saline (pH=6.9) and pre-incubated for 15 min at 37 °C. Volume 20 μL of potato starch (1 % *W/V* in 20 mM phosphate buffered saline pH=6.9) was added after the pre-incubation. Final concentration of predigested spinach lemon juice formulations used was 6 mg/mL and α-amylase was 1 unit/mL. Lemon juice was also diluted to final concentration of 4 %. After 15 min of incubation at 37 °C, 50 μL of dinitrosalicylic acid reagent (12 mL of distilled water, 8 mL of 5.3 M potassium sodium tartrate, tetrahydrate solution in 2 M NaOH and 20 mL of 96 mM 3,5-dinitrosalicylic acid solution) was added and incubated at 85 °C for 15 min. Volume 450 μL of distilled water was mixed with tested solution and the absorbance was measured at 545 nm. Appropriate blanks and controls were carried out. The α-amylase enzyme inhibitory activity (%) was calculated from the equation:

$$\text{amylase inhibition}=100-((A_{sample}-A_{sample\ blank})/(A_{control}-A_{control\ blank})\cdot 100) \quad /3/$$

where A_{sample} was the absorbance of the test (with amylase), $A_{sample\ blank}$ was the absorbance of test blank (without amylase), $A_{control}$ was the absorbance of control (with amylase) and $A_{control\ blank}$ was the absorbance of control blank (without amylase). Maltose was used as a positive control. All absorbance measurements were performed with microplate reader Fluostar Optima (BMG Labtech GmbH, Offenburg, Germany).

Bioaccessibility (BC) of phytochemicals

The bioaccessibility (%) was calculated from the equations:

$$\text{Bioaccessibility in salivary phase}=(BC_{salivary\ phase}/BC_{initial\ phase})\cdot 100 \quad /4/$$

$$\text{Bioaccessibility in gastric phase} = (BC_{\text{gastric phase}}/BC_{\text{initial phase}}) \cdot 100 \quad /5/$$

$$\text{Bioaccessibility in intestinal phase} = (BC_{\text{intestinal phase}}/BC_{\text{initial phase}}) \cdot 100 \quad /6/$$

where $BC_{\text{salivary phase}}$, $BC_{\text{gastric phase}}$ and $BC_{\text{intestinal phase}}$ corresponded to the total or individual bioactive compound concentration in salivary, gastric and intestinal phase and BC_{initial} was the total or individual bioactive compound concentration in initial phase.

Statistical analysis

All results were evaluated using STATISTICA version 14.0.0.15 software (TIBCO Software Inc., Palo Alto, CA, USA) (*31*). RP-HPLC and results from spectrophotometric determination were subjected to one-way ANOVA for comparison of means and significant differences were calculated according to Duncan's multiple range test. The data are presented as means ±standard deviations (S.D.). Pearson's correlation coefficient and principle component analysis (PCA) between phytochemicals, antioxidant and antidiabetic activity was performed. Data were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Phytochemical analysis

TP, TF, TPA, OA and nitrate content of predigested and digested spinach lemon juice formulations are presented in Table 1. Significant increases in TP content were noted after addition of 20 % lemon juice in predigested and almost all digested spinach samples with the exception of TP in initial phase of digestion. During that phase significant increases were noted in spinach samples with 10 % lemon juice in comparison to spinach formulated with 0 % of lemon juice. TF and TPA content were significantly higher in all predigested spinach samples with lemon juice in comparison to the predigested spinach sample (S) without lemon juice. The highest TP content was detected in predigested spinach samples with 20 % lemon juice and in salivary and gastric phase, while the highest TPA content was detected in predigested and all phases of digestion. Significant increase of total polyphenolic groups in predigested spinach lemon juice formulation can be due to lemon juice acidity which affected stability of tested total polyphenolic groups in acidic solutions (*17*). This phenomenon can best be seen in the spinach lemon formulation in which the highest percentage of lemon juice is added. The range of TP, TF and TPA content was 8.47-15.73 mg/g dm, 6.32-9.19 mg/g dm and 1.82-4.53 mg/g dm, respectively. These results for TP were within the range reported by Turkmen *et al.* (*32*) and higher than reported by Bunea *et al.* (*33*) and Roberts and Moreau *et al.* (*2*) for fresh, refrigerated processed spinach or dried spinach extract. Roberts and Moreau (*2*) also reported lower values of TF and TPA for fresh spinach. OA content was statistically

higher in spinach lemon juice formulation (2, 10 and 20 % for original samples; 5, 10 and 20 % for initial phase and 2, 5, 10 and 20 % for salivary, gastric and intestinal phase) in a dose depending manner when compared to spinach samples (S). The range of OA content was 6.02-12.24 mg/g dm. Similar values were recorded by Akhtar *et al.* (*34*) and Savage *et al.* (*35*) for raw or boiled spinach and they were ten times less than Albert *et al.* (*36*) recorded for dried spinach water extract. Average oxalate concentration in this study was 8.97 mg/g dm whereas Wang *et al.* (*4*) observed higher average oxalate concentration 20.76 mg/g fm. According to Akhtar *et al.* (*34*) raw spinach contains about 55 % soluble and 45 % insoluble (mostly calcium) oxalates and both can be significantly reduced after boiling. Boiling can result in 50 % of oxalic acid reduction (*5*). Insoluble oxalates were removed from our spinach water extract by filtration process (Whatman Grade 595, pore size 4-7 μm). Boiling and filtration processes may be the reason why we assessed lower oxalic acid values in spinach lemon juice formulation. Significanly higher nitrate content was noted in spinach samples with 5, 10 and 20 % lemon juice addition in predigested form and in spinach sample with 20 % addition of lemon juice in digested (salivary phase and intestinal phase) spinach samples in comparison to S. Nitrate content was in range of 7.41-20.49 mg/g dm. Average nitrate concentration in our study was 14.10 mg/g dm. Ten times lower average values of dietary nitrate were observed by Wang *et al.* (*4*) in fresh spinach. Morgado *et al.* (*7*) reported ten times lower average values of TP and 1.6 and 2.3 lower nitrate values in nitrate rich beetroot gel and juice before and after *in vitro* digestion, respectively. Higher values of TP and nitrate before and after *in vitro* digestion in our study were most likely obtained due to the fine spinach powder usage which is supported by the results found in Prasaedaya *et al.* (*24*) study. According to that group of authors smaller particle size have positive impact on bioactive compounds extraction. When we observe the mean values of TP of all treatments, we can see the highest content of TP in the oral phase of *in vitro* digestion and a no significant decrease of TP in the gastric, significant decrease in initial and then in intestinal phase and the lowest values in the original samples. Spinach formulated with 20 % of lemon juice had the highest TP values. Accordingly, lemon juice can increase stability of spinach TP in spinach with 20% lemon juice formulation. Highest TF content of mean values of all treatments was observed before digestion (original samples) and in initial and salivary phase of digestion and significant drop was observed for gastric and intestinal phase. Same trend was observed for spinach formulated with 20 % of lemon juice in predigested and digested samples. In other formulations the stated stability of TF was not observed. When we observe the mean values of OA of all treatments and spinach formulated with 10 % of lemon juice, we can see the same trend for mean values for TF in spinach lemon juice formulation. The highest mean values of TPA of all treatments were observed in initial and salivary phase, then significant drop was observed in intestinal, then in gastric phase and the

smallest values od TPA was detected before *in vitro* digestion in original samples. This trend was observed in almost all individual spinach lemon juice formulation. A significant gradual decline in nitrate content from the initial to the intestinal phase in the mean values of all treatments is visible and the smallest values of nitrate was reported in original samples. Wootton-Beard *et al.* (*37*) detected increase in TP content after gastric phase in all 23 tested vegetable juices and decrease or increase after intestinal phase. Few of Wootton-Beard *et al.* (*37*) vegetable juices in their composition have lemon juice and spinach. Morgado *et al*. (*7*) detected increase in TP content after gastric phase and duodenal phases and increase in nitrate content after gastric and decrease of nitrate content in intestinal phase of *in vitro* digestion. Significant increase of TP of Matcha tea was observed in gastric phase of *in vitro* digestion and then decrease in intestinal phase (*17*). For TF the highest values of Matcha tea were observed in initial and salivary phase and significant drop was observed for gastric and then for intestinal phase (*17*). TP, TF, TPA, OA and nitrate content of lemon juice is presented in Table 2. Content of TP, TF, TPA and nitrate of pure lemon juice was 2.95, 1.95, 3.49 and 13.23 times smaller than predigested spinach sample (S), respectively. Lemon juice is by 1.32 times richer source of oxalate by than predigested spinach samples. According to the data, addition of lemon juice does not contribute to the contents of TP, TF, TPA and nitrate in spinach lemon juice formulation, but contributes to the content of the OA in spinach lemon juice formulation in dose depending manner. But lemon juice can accelerate polyphenol extraction due to the change in pH of the extraction solvent (*38*). We reported significant decrease of pH in dose depended manner for predigested lemon juice formulation (0, 2, 5, 10 and 20 %) from 5.06, 4.65, 4.04, 3.83 to 3.34 (Table S1). This pH trend (Table S1) was also observed for initial (6.66, 6.60, 6.13, 5.29 and 4.45), salivary (6.82, 6.65, 6.16, 5.28 and 4.39), gastric (2.24, 2.10, 2.04, 1.94 and 1.78) and intestinal (7.04, 6.70, 6.64, 6.30 and 6.12) phase of digestion. Pure lemon juice pH was 2.35 (Table 2) and was statistically lower (2.15 times) than predigested spinach sample (S). According to results of pH and total polyphenolic groups, pH of lemon juice can positively influence the content of polyphenolic groups.

Concentration of *L*-AA and individual phenolic compounds (*p*-CA, FA and Q) in predigested spinach lemon juice formulations is presented in Fig. 1. There was no significant increase in *L*-AA (Fig. 1a) and *p*-CA (Fig. 1b) in all the tested predigested spinach lemon juice formulations. The highest concentration of FA (Fig. 1c) was observed in spinach predigested samples with addition of 5 % of lemon juice and the smallest concentration of FA was observed in the spinach lemon juice formulation with 0 and 20 % of lemon juice. Concentration of Q (Fig. 1d) was significantly higher in spinach lemon juice formulations (2, 5 and 10 %) compared to spinach lemon juice formulation with 0 and 20 %. *L*-AA, *p*-CA, FA and Q concentrations of lemon juice are presented in Table 2. Lemon

juice is richer source of *L*-AA by 1.07 time than predigested spinach samples. Concentration of all individual phenolic compounds (*p*-CA, FA and Q) was 24.01, 7.51 and 1.01 times lower than in predigested spinach sample, respectively. Accordingly, the addition of lemon juice does not contribute to the contents of individual phenolic compounds (*p*-CA, FA and Q), but contributes to the content of the *L*-AA in spinach lemon juice formulation. RP-HPLC analysis of *L*-AA and individual phenolic compounds (*p*-CA, FA and Q) in digested were measured only in 0 and 20 % spinach lemon juice formulations (Fig. 2). Significant increases in concentration of *L*-AA (Fig. 2a) was noted in salivary phase in spinach lemon juice formulated with 20 % of lemon juice when analyzed for each phase individually. When data were analyzed for all samples and phases together significant increase of *L*-AA was detected in initial and intestinal phase of digestion. Addition of lemon juice to spinach increased *L*-AA concentration in initial, salivary, gastric and intestinal phase of digestion for 1.18, 1.10, 1.10 and 1.15 times compared to lemon juice formulated with 0 % of lemon juice, respectively. Concentration of *p*-CA (Fig. 2b) significantly increased in the initial phase and Q (Fig. 2d) in the intestinal phase of digestion in spinach lemon juice formulated with 20 % when analyzed for each phase individually. When data were analyzed for all samples and phases together significant decrease of *p*-CA (Fig. 2b) was observed in gastric phase of digestion in spinach lemon juice formulated with 20 % of lemon juice. There was no significant increase in FA (Fig. 2c) concentration in all phases of *in vitro* digestion between spinach formulation with 0 and 20 % of lemon juice. In predigested and digested spinach samples concentration of *L*-AA, *p*-CA, FA and Q was in range of 1272.79-4200.19 mg/kg dm, 140.94-179.43 mg/kg dm, 121.59-168.45 mg/kg dm, 30.91-123.98 mg/kg dm, respectively. In the study of Delchier *et al.* (*39*), concentration of vitamin C in spinach samples varied from 74 to 170 mg/kg after boiling. These results for vitamin C were about twenty times smaller than in our study. Bunea *et al.* (*33*) detected lower concentration of *o*-coumaric acid (28–60 mg/kg fm), ferulic acid (10–35 mg/kg fm) and *p*-coumaric acid (1–30 mg/kg fm) in fresh, refrigerated and processed spinach. They reported an increase in concentration for all phenolic acids after boiling procedure due to the degradation of complex phenolic structures (tannins or flavonoids) into simple phenolics (phenolic acids). According to Roberts and Moreau (*2*) spinach contains negligible amounts of quercetin and kaempferol. High values of *L*-AA, nitrate, total and individual phenolic compounds in comparison with the results presented by other authors can be due to dry fine spinach powder usage for sample preparation. In our sample the preparation was done by cooking process (40 min), during which the high temperature can destroy the cell walls of the dry spinach powder and release phytochemicals from the food matrix.

Phytochemical bioaccessibility

Bioaccessibility of total and individual polyphenol, *L*-AA, OA and nitrate are presented in Table 3. The mean values for the bioaccessibility percentage point out the decline through *in vitro* digestion for OA and nitrate. For TF and Q the highest mean values of bioaccessibility were obtained in salivary phase and the significant drop was observed in gastric and intestinal phase of *in vitro* digestion. No significant change was observed for *L*-AA and *p*-CA bioaccessibility mean values. Significant drop for TP and FA bioaccessibility mean values was observed in intestinal phase as opposed to the salivary phase. For TPA the highest mean values of bioaccessibility was measured in salivary phase and the lowest in gastric phase of digestion. High bioaccessibility of TPA in intestinal phase can be due to degradation of complex polyphenols to simple phenolic acids after hydrolysis in gastric phase (acid environment pH=2) of digestion. Bioaccessibility were very high for all parameters and in the range of 75.72-139.51 % for TP, 75.93-106.20 % for TF, 73.41-105.88 % for TPA, 86.19-104.56 % for *p*-CA, 92.50-108.83 % for FA and 98.06-101.46 % for Q, 100.78-108.98 % for *L*-AA, 64.72-100.97 % for oxalic acid and 51.54-92.90 % for nitrate. The highest bioaccessibility of TP (132.08 % for salivary phase, 139.51 % for gastric phase and 105.85 % for intestinal phase) were observed in spinach lemon juice formulated with highest concentration of lemon juice. In spinach samples formulated with 20 % of lemon juice the highest percentage of bioaccessibility for TPA in gastric (85.14) and intestinal (97.36) phase and for nitrate (60.16) an Q (100.11) in intestinal phase of digestion. The highest bioaccessibility of OA (100.97 % for salivary phase, 87.40 % for gastric phase and 68.47 % for intestinal phase) was observed in spinach formulated with 5 % of lemon juice. Significantly higher *L*-AA bioaccessibility (107.72 %) for salivary phase was observed in spinach formulated with 0 % of lemon juice in comparison to spinach formulated with highest concentration of lemon juice. Wootton-Beard *et al*. (*37*) also detected high bioaccessibility of TP of few vegetable juices which in their composition had lemon juice and spinach. Green *et al.* (*14*) detected significant increase in total catechin recovery in green tea formulated with 20 % and 50 % of lemon juice. Small percentage of the observed enhancement in digestive recovery of catechins in tea-juice formulations was achieved by ascorbic acid from lemon juice. Bravo (*40*) reported that quercetin and free simple phenolic acids are directly absorbed through the small intestinal mucosa and Iqbal *et al.* (*41*) reported that *L*-AA is absorbed in the buccal mucosa, stomach and the small intestine. Accordingly, all spinach lemon juice formulation represents a good source of high intestinal bioaccessible Q and individual phenolic acids and high salivary, gastric and intestinal bioaccessible *L*-AA. Morgado *et al.* (*7*) also detected high percentage of nitrate bioaccessibility in nitrate rich beetroot gel and juice. Nitrate from green leafy vegetables or beetroot is mainly reabsorbed in the mouth (in the salivary glands) and then in the proximal intestines (*8)*. Bioaccessibility of nitrate in

spinach lemon juice formulation was very high (81.70-92.90 %) in the salivary phase for all samples and the highest (60.16 %) in the intestinal phase in spinach formulated with highest lemon concentration (20 %). Therefore, spinach formulated with 20 % of lemon juice represent best source of dietary nitrate. Savage and Catherwood (*42*) reported that oxalate is absorbed by small intestine or in the ileum. Bioaccessibility of oxalate in spinach lemon juice formulation was in the range of 64.72-68.47 % for intestinal phase of *in vitro* digestion. This percentage of oxalate bioaccessibility can be drastically reduced in human intestine depending on the co-ingestion of calcium magnesium and fibre (*42*).

Antioxidant activity

Antioxidant activity of predigested and digested spinach lemon juice formulations was estimated with DPPH and FRAP (Figs. 3a−3d). According to DPPH method when results are expressed in percentage (Fig. 3a) all predigested spinach lemon juice formulations and pure lemon juice (Table 2) samples have a strong antioxidant activity in relation to the Trolox (88 %). Strong antioxidant activity, detected with DPPH method (Fig. 3a), was noted also in initial phase and salivary phase in spinach samples with 20 % lemon juice. All predigested and digested spinach lemon juice formulations and pure lemon juice samples have a strong antioxidant activity, detected with FRAP method, expressed in percentage (Fig. 3b) in relation to the Trolox (97 %). Statistically the highest antioxidant activity in both antioxidant methods expressed in percentage and in μM (Figs. 3c and 3d) was noted in predigested and all digested spinach samples with 20 % lemon juice. In almost all predigested and digested spinach lemon juice formulation (2, 5, 10 and 20 %) there was significant increase in both antioxidant methods expressed in percentage and μM in dose depending manner in comparison to S. We can observe increase in antioxidant activity in gastric phase and decrease after intestinal phase compared to salivary phase in almost all spinach lemon juice formulations for FRAP method (Fig b and d) and in some spinach lemon juice formulations for DPPH method (Figs. 3a and 3c). This trend was also observed by Wootton-Beard *et al.* (*37*) in DPPH and FRAP methods and Rusak *et al.* (*17*) in ABTS, DPPH and FRAP methods for Matcha tea. We assumed that an acid environment in gastric digestion (pH=2) positively affects polyphenols which are known antioxidants. With Pearson correlation coefficients we confirmed this claim (Table S2). All total polyphenols (TP, TF and TPA) and antioxidant activity methods (expressed in percentage and in μM) significantly correlated to each other in gastric phase of digestion and had very strong (0.801-0.941) or strong correlations (0.634-0.795). This trend was observed for almost all antioxidant activity methods in initial and intestinal phase of digestion for TP and TPA. Very strong (-0.822-0.964) or strong negative correlations (-0.773-0.788) in gastric phase between pH and total polyphenols, oxalic acid, nitrate

and antioxidant activity was obtained (Table S2). Negative correlations between pH and TP (0.748), TPA (0.946), OA (0.966), nitrate (0.714), DPPH (%: 0.976, μM: 0.973) and FRAP (%: 0.932, μM: 0.980) were observed for initial phase (Table S2). Negative correlation between pH and TPA (0.714), OA (0.747), DPPH (%: 0.902, μM: 0.904) and FRAP (μM: 0.756) was observed for intestinal phase of digestion (Table S2). This means that lowering the pH of spinach lemon juice formulation contributes to increasing the content of phytochemicals and antioxidant activity. Based on these correlations we can conclude that oxalic acid affects pH. Oxalic acid is relatively strong acid which may function as pH regulator in plants (*44*). Almost all antioxidant activity methods correlated very strongly (>0.828-0.988) or strongly (0.718-0.769) with oxalic acid and strongly with nitrate in initial, gastric and intestinal phase of digestion. According to the literature oxalic acid can act as a natural antioxidant by reducing the rate of ascorbic acid oxidation in the presence of hydrogen peroxide and Cu^{2+}(*44*) and dietary nitrate can have positive effect on antioxidant capacity in animal model (*45*). According to the literature, both spinach and lemon juice are good source of antioxidant (*2,11,14,32,43*). Antioxidant activity with both methods (DPPH %, DPPH μM, FRAP % and FRAP μM) of pure lemon juice (Table 2) was 1.25, 1.10, 1.02 and 3.55 time higher than predigested S, respectively (Figs. 3a−3d).

α-amylase inhibitory activity

Antidiabetic properties of predigested spinach lemon juice formulations and lemon juice through inhibition of α-amylase is presented in Fig. 4 and in Table 2. Strong α-amylase inhibitory activity was observed only in spinach samples with 20 % lemon juice (75.02 %) and in diluted lemon juice (81.94 %) in relation to the maltose (90 %) (Fig. 4). All other spinach lemon juice formulations had a weak α-amylase inhibitory activity of 20.02, 21.48, 25.06 and 27.52, respectively in relation to the maltose. Statistically significant increase of percentage of inhibition of α-amylase was measured in 5, 10 and 20 % spinach lemon juice formulation in dose depending manner in comparison to S. It seems that the addition of lemon juice in lemon juice formulation contributes to α-amylase inhibitory activity in spinach lemon juice formulation in dose depending manner. α-amylase inhibitory activity of pure lemon juice (Table 2) was 4.09 time higher than predigested S (Fig. 4). Gironés-Vilaplana *at al.* (*6*) reported that lemon and lime have good antidiabetic activity, which correlated with vitamin C and flavone contents. Rusak *et al.* (*17*) reported that antioxidant and antidiabetic activity depend on the concentration of total and individual polyphenolic compounds before and during *in vitro* digestion. In predigested samples (Table S3) α-amylase inhibitory activity correlated very strongly (>0.800) or strongly (>0.600<0.799) with antioxidant activity (FRAP %; 0.859, FRAP μM; 0.975, DPPH %; 0.645 and DPPH μM; 0.811), TP (0.755), TPA (0.774), OA (0.797) and nitrate (0.646). Khalifi *et al.* (*45*)

reported positive outcomes of dietary nitrate in type 2 diabetic rats on glucose tolerance and antioxidant capacity Very strong (-0.811-0.970) or strong negative correlations (-0.738-0.780) in predigested samples between pH and almost all total polyphenols, oxalic acid, nitrate and antioxidant activity and moderate negative correlation (-0.651) between pH and α-amylase inhibitory activity were obtained (Table S3). Accordingly, low pH can positively influence the extraction of phytochemicals that have strong positive effect on antioxidant and moderate effect on antidiabetic activity.

PCA

The PCA plots provide an overview of the similarities and differences between different spinach lemon juice formulation as well as the interrelationships between the measured properties (pH, phytochemical composition, antioxidant and antidiabetic activity) (Figs. 5a−5d). The first (PC 1) and the second principal component (PC2) described 63.74 % and 25.21 % of the variance for predigested samples (Fig. 5a), 70.92 % and 26.92% of the variance for digested - salivary phase (Fig. 5b), 64.52 % and 31.75 % of the variance for digested - gastric phase (Fig. 5c) and 61.87 % and 23.74 % of the variance for digested - intestinal phase (Fig. 5d). Together, the first two PCs represent 88.95 % of the total variability for predigested (Fig. 5a), 97.84 % for digested – salivary (Fig. 5b), 96.27 % for digested – gastric phase (Fig. 5c) and 96.61 % for digested – intestinal phase (Fig. 5d). The highest distance which points to the biggest difference was detected between spinach formulated with 0% of lemon juice and spinach formulated with 20% of lemon juice in predigested and in all digested samples (Figs. 5a−5d). The smallest distance which points to the smallest difference was detected between spinach lemon juice formulation (2, 5, 10 %) in predigested (Fig. 5a) and in digested samples (Figs. 5b−5d). Spinach formulated with 20 % of lemon juice had strong loadings with most tested total (TP, TPA, OA and nitrate) compounds, antioxidant and α-amylase inhibitory activity in predigested stage (Fig. 5a). In the above mentioned methods spinach formulated with highest percentage of lemon juice had the highest mean values compared to all other spinach lemon formulation. Spinach formulated with 0 % of lemon juice had strong loadings with *L*-AA and pH and this sample has the highest measured values of *L*-AA and pH compared to other formulations. The highest distance which points to the biggest difference was detected between *L*-AA and pH, and all other methods in predigested samples. These results indicate that total polyphenolic compounds, oxalate and nitrate in predigested samples are closely related to antioxidant activity and α-amylase inhibitory activity and that lowering the pH value with lemon juice can positively affect these parameters which is confirmed by the literature (*17,38,44,45*). In all digested samples (Figs. 5b−5d) spinach formulated with highest concentration of lemon juice had

strong loadings with most tested total polyphenolics, antioxidant methods, nitrate, oxalate and individual compounds measured by HPLC (*L*-AA, *p*-CA, FA and Q) and spinach formulated with 0 % of lemon juice had strong loadings with pH. Accordingly, polyphenolic compounds and *L*-AA in almost all digested samples are closely related with antioxidant activity which is consistent with the literature (*25*). The highest distance was detected between pH and all other methods in digested samples. This means that by reducing the pH by adding lemon juice in higher concentrations contributes to the stabilization of polyphenols and vitamin C in *in vitro* digestion, which directly affects the increase of antioxidant activity of spinach formulation.

CONCLUSIONS

This is the first report about stability and digestive availability of spinach polyphenols, oxalic acid, nitrate and *L*-ascorbic acid in spinach lemon formulations. High bioavailability of almost all tested compounds can be attributed to the preparation of samples with fine powder and concentration of the sample by cooking for 40 minutes, during which the high temperature can destroy the cell walls of the dry spinach powder and release phytochemicals from the food matrix. Lemon juice pH and antioxidants (*L*-AA,) increase stability and availability of polyphenols in spinach lemon juice formulation during *in vitro* digestion and positively influence antioxidant activity. Addition of lemon juice in lemon juice formulation contributes to α-amylase inhibitory activity in dose depending manner. Spinach formulated with 20 % of lemon juice represents the best source of dietary polyphenols and nitrate with antioxidant and antidiabetic activity that may be used as functional drink for health and exercise performance improvement.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

VVB conceived, carried out the experiments and wrote the MS; IŠ carried out HPLC analysis, GR supervised the work and edited the manuscript.

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Table 1. The total polyphenols, total flavonoids, total phenolic acids, oxalic acid and nitrate content in predigested (original sample) and digested (initial phase, salivary phase, gastric phase and intestinal phase) spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

w(TP as GAE)/(mg/g dm)					
Sample	Original sample	Initial phase	Salivary phase	Gastric phase	Intestinal phase
S	(8.60±0.29)^{bC}	(10.39±0.74)^{bABC}	(11.60±0.85)^{bA}	(10.92±2.14)^{bcAB}	(9.55±0.79)^{bBC}
S + 2 % L	(8.47±0.52)^{bC}	(10.78±0.80)^{abAB}	(12.16±1.61)^{bA}	(9.75±1.21)^{cBC}	(9.32±1.08)^{bBC}
S + 5 % L	(8.79±0.62)^{abC}	(11.03±1.00)^{abAB}	(12.26±1.20)^{bA}	(11.79±1.43)^{bcA}	(9.94±0.84)^{abBC}
S+ 10 % L	(8.83±0.67)^{abB}	(12.44±1.05)^{aA}	(13.34±0.80)^{abA}	(13.62±2.95)^{abA}	(9.42±2.10)^{bB}
S + 20 % L	(9.60±0.73)^{aC}	(11.27±1.69)^{abB}	(14.89±1.57)^{aA}	(15.73±0.80)^{aA}	(11.93±1.39)^{aB}
Mean	(8.86±0.66)^{D}	(11.26±1.20)^{B}	(12.85±1.63)^{A}	(12.18±2.67)^{AB}	(10.03±1.55)^{C}
w(TF as QE)/(mg/g dm)					
Sample	Original sample	Initial phase	Salivary phase	Gastric phase	Intestinal phase
S	(6.32±2.27)^{bB}	(8.75±0.11)^{aA}	(7.96±0.09)^{bAB}	(6.82±0.11)^{bcB}	(7.36±0.30)^{aAB}
S + 2 % L	(8.85±0.09)^{aA}	(7.89±0.12)^{bB}	(8.02±0.03)^{bB}	(6.68±0.14)^{cC}	(6.76±0.07)^{bcC}
S + 5 % L	(9.10±0.25)^{aA}	(7.67±0.14)^{bB}	(7.81±0.40)^{bB}	(6.97±0.09)^{bcC}	(6.46±0.14)^{cC}
S + 10 % L	(9.06±0.19)^{aA}	(8.83±0.68)^{aA}	(8.07±0.05)^{bB}	(7.10±0.40)^{bC}	(6.71±0.36)^{bcC}
S + 20 % L	(8.92±0.08)^{aA}	(8.65±0.39)^{aA}	(9.19±1.06)^{aA}	(7.63±0.08)^{aB}	(7.06±0.38)^{abB}
Mean	(8.45±1.43)^{A}	(8.36±0.59)^{A}	(8.21±0.68)^{A}	(7.04±0.38)^{B}	(6.86±0.38)^{B}
w(TPA as CAE)/(mg/g dm)					
Sample	Original sample	Initial phase	Salivary phase	Gastric phase	Intestinal phase
S	(1.82±0.10)^{dE}	(3.55±0.06)^{dB}	(3.76±0.11)^{dA}	(2.94±0.10)^{cD}	(3.24±0.18)^{cC}
S + 2 % L	(2.20±0.06)^{bD}	(4.11±0.27)^{bcA}	(3.85±0.10)^{cdA}	(3.01±0.23)^{cC}	(3.45±0.19)^{cB}
S + 5 % L	(2.09±0.03)^{cD}	(3.92±0.14)^{cA}	(3.99±0.12)^{bcA}	(3.10±0.11)^{bcC}	(3.44±0.08)^{cB}
S + 10 % L	(2.12±0.03)^{bcD}	(4.28±0.32)^{abA}	(4.16±0.14)^{bA}	(3.34±0.25)^{bC}	(3.78±0.21)^{bB}
S + 20 % L	(2.30±0.04)^{aC}	(4.49±0.26)^{aA}	(4.53±0.21)^{aA}	(3.82±0.17)^{aB}	(4.37±0.15)^{aA}
Mean	(2.11±0.17)^{D}	(4.07±0.39)^{A}	(4.06±0.31)^{A}	(3.24±0.36)^{C}	(3.66±0.36)^{B}
w(oxalic acid content asOAE)/(mg/g dm)					
Sample	Original sample	Initial phase	Salivary phase	Gastric phase	Intestinal phase
S	(7.46±0.95)^{dB}	(9.30±0.01)^{dA}	(9.22±0.06)^{eA}	(7.98±0.01)^{eB}	(6.02±0.07)^{eC}
S + 2 % L	(9.24±0.24)^{cB}	(9.47±0.01)^{cdA}	(9.36±0.10)^{dAB}	(8.11±0.03)^{dC}	(6.31±0.04)^{dC}
S + 5 % L	(8.41±1.09)^{cdB}	(9.54±0.29)^{cA}	(9.63±0.03)^{cA}	(8.34±0.01)^{cB}	(6.53±0.02)^{cC}
S+ 10 % L	(10.35±0.58)^{bA}	(10.23±0.01)^{bA}	(10.19±0.05)^{bA}	(8.80±0.01)^{bB}	(6.90±0.06)^{bC}
S + 20 % L	(12.24±0.01)^{aA}	(11.54±0.02)^{aB}	(11.53±0.01)^{aB}	(9.91±0.01)^{aC}	(7.71±0.06)^{aD}
Mean	(9.54±1.81)^{A}	(10.02±0.85)^{A}	(9.98±0.86)^{A}	(8.63±0.72)^{B}	(6.690±0.60)^{B}
w(nitrate content as KNO_3E)/(mg/g dm)					
Sample	Original sample	Initial phase	Salivary phase	Gastric phase	Intestinal phase
S	(7.41±0.23)^{cE}	(19.67±0.09)^{abA}	(16.07±1.06)^{bB}	(13.08±0.34)^{cC}	(10.21±0.54)^{bD}
S + 2 % L	(8.16±0.16)^{bcE}	(19.33±0.26)^{bA}	(16.68±0.93)^{abB}	(13.48±0.45)^{bcC}	(9.96±0.43)^{bD}
S + 5 % L	(9.21±0.06)^{abD}	(19.51±0.27)^{bA}	(17.17±1.17)^{abB}	(13.50±0.17)^{abcC}	(10.61±0.30)^{bD}

S + 10 % L	(9.85±1.17)^{aC}	(20.16±0.34)^{abA}	(16.79±1.30)^{abB}	(14.40±0.15)^{aB}	(10.49±1.07)^{bC}
S + 20 % L	(10.42±0.17)^{aE}	(20.49±0.51)^{aA}	(19.04±0.39)^{aB}	(14.37±0.46)^{abC}	(12.33±0.25)^{aD}
Mean	(9.01±1.22)^{E}	(19.83±0.51)^{A}	(17.15±1.31)^{B}	(13.77±0.61)^{C}	(10.72±0.98)^{D}

Data are presented as mean value±S.D, N=4.Different small letters indicate significant difference at p≤0.05 for each phase separately. Capital letters indicate significant difference at p≤0.05 between all phases together S=spinach, L=lemon juice, TP=total polyphenols, GAE=gallic acid equivalents, TF=total flavonoids, QE=quercetin equivalent, TPA=total phenolic acids, CAE=caffeic acid equivalents, OAE=oxalic acid equivalents, KNO_3E=KNO_3 equivalents.

Table 2. Phytochemical content, antioxidant activity ,antidiabetic activity and pH of pure lemon juice

w(TP as GAE)(mg/g dm)	*w*(oxalic acid content as OAE)/(mg/g dm)	*w*(*p*-CA)/(mg/kg dm)	DPPH inhibition/%	FRAP reduction/%
2.91±0.12	9.82±0.02	5.87±0.09	80.23±1.79	93.39±0.34
w(TF as QE)/(mg/g dm)	*w*(nitrate content as KNO_3E)/(mg/g dm)	*w*(FA)/(mg/kg dm)	DPPH as TE/(μM /g dm)	FRAP as Fe^{2+}E/(μM/g dm)
3.22±0,11	0.56±0.05	16.19±0.65	2641.99±60.18	3250.79±180.23
w(TPA as CAE)/(mg/g dm)	*w*(*L*-AA)/(mg/kg dm)	*w*(Q)/(mg/kg dm)	α-amylase inhibition/%	pH
0.52±0.02	1519.69±111.68	30.51±0.73	81.94±6.02	2.35±0.02

Data are presented as mean value±S.D, N=4. TP=total polyphenols, GAE=gallic acid equivalents, TF=total flavonoids, QE=quercetin equivalent, TPA=total phenolic acids, CAE=caffeic acid equivalents, OAE=oxalic acid equivalents, KNO_3E=KNO_3 equivalents, *L*-AA=*L*-ascorbic acid, *p*-CA=*p*-coumaric acid, FA=ferulic acid, Q=quercetin, TE=Trolox equivalents, Fe^{2+}E= Fe^{2+} equivalents

Table 3. Bioaccessibility of total and individual polyphenols oxalic acid , nitrate and *L*-ascorbic acid in spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

TP bioaccessibility/%			
Sample	Salivary phase	Gastric phase	Intestinal phase
S	$(111.64±8.18)^{abA}$	$(105.17±20.63)^{abA}$	$(91.92±7.57)^{abA}$
S + 2 % L	$(112.83±14.91)^{abA}$	$(90.44±11.19)^{bB}$	$(86.45±10.03)^{abB}$
S + 5 % L	$(111.22±10.88)^{abA}$	$(106.90±12.99)^{abAB}$	$(90.18±7.65)^{abB}$
S + 10 % L	$(107.29±6.42)^{bA}$	$(109.50±23.74)^{abA}$	$(75.72±16.87)^{bB}$
S + 20 % L	$(132.08±13.31)^{aA}$	$(139.51±6.77)^{aA}$	$(105.85±11.78)^{aB}$
Mean	$(115.01±8.74)^{A}$	$(110.30±16.04)^{A}$	$(90.02±9.71)^{B}$

TF bioaccessibility/%				TPA bioaccessibility/%			
Sample	Salivary phase	Gastric phase	Intestinal phase	Sample	Salivary phase	Gastric phase	Intestinal phase
S	$(90.90±1.02)^{bA}$	$(77.85±1.21)^{cC}$	$(84.06±1.02)^{aB}$	S	$(105.88±3.18)^{aA}$	$(82.75±2.77)^{abC}$	$(91.44±4.98)^{abB}$
S + 2 % L	$(101.61±0.36)^{aA}$	$(84.58±1.71)^{bB}$	$(85.65±1.02)^{aB}$	S + 2 % L	$(93.74±2.40)^{cA}$	$(73.41±5.55)^{cC}$	$(83.97±4.51)^{cB}$

S + 5 % L	(101.87±5.24)^{aA}	(90.87±1.20)^{aB}	(84.21±1.02)^{aC}	S + 5 % L	(101.80±3.13)^{abA}	(79.12±2,79)^{abcC}	(87.96±1.92)^{bcB}
S +10 % L	(91.35±0.62)^{bA}	(80.37±4.56)^{cB}	(75.93±1.02)^{bB}	S + 10 % L	(97.16±3.20)^{bcA}	(77.85±8.83)^{bcC}	(88.28±4.85)^{bcB}
S + 20 % L	(106.20±12.25)^{aA}	(88.15±0.88)^{aB}	(81.60±1.02)^{aB}	S + 20 % L	(100.86±4.58)^{abA}	(85.14±3.75) ^{aB}	(97.36±3.25)^{aA}
Mean	(98.39±6.15)^{A}	(84.37±4.80)^{B}	(82.29±3.43)^{B}	Mean	(99.89±4.14)^{A}	(79.65±4.05)^{C}	(89.80±4.46)^{B}
OA bioaccessibility/%				Nitrate bioaccessibility/%			
Sample	Salivary phase	Gastric phase	Intestinal phase	Sample	Salivary phase	Gastric phase	Intestinal phase
S	(99.12±0.65)^{bcA}	(85.86±0.05)^{bcB}	(64.72±0.70)^{dC}	S	(81.70±5.41)^{aA}	(66.48±1.72)^{bB}	(51.93±2.75)^{bC}
S + 2 % L	(98.78±1.08)^{cA}	(85.64±0.29)^{cB}	(66.58±0.39)^{cC}	S + 2 % L	(86.29±4.82)^{aA}	(69.73±2.35)^{abB}	(51.54±2.24)^{bC}
S + 5 % L	(100.97±0.27)^{aA}	(87.40±0.15)^{aB}	(68.47±0.25)^{aC}	S + 5 % L	(88.01±5.97)^{aA}	(69.20±0.85)^{abB}	(54.36±1.55)^{bC}
S + 10 % L	(99.55±0.45)^{bcA}	(85.94±0.10)^{cB}	(67.47±0.59)^{bC}	S + 10 % L	(83.27±6.46)^{aA}	(71.45±0.75)^{aA}	(52.06±5.31)^{abB}
S + 20 % L	(99.92±0.08)^{bcA}	(85.87±0.07)^{bcB}	(66.85±0.55)^{bcC}	S + 20 % L	(92.90±1.89)^{aA}	(70.14±1.72)^{abB}	(60.16±1.23)^{aC}
Mean	(99.67±0.76)^{A}	(86.14±0.64)^{B}	(66.82±1.23)^{C}	Mean	(86.44±3.92)^{A}	(69.40±1.64)^{B}	(54.01±3.23)^{C}
L-AA bioaccessibility/%				*p*-CA bioaccessibility/%			
Sample	Salivary phase	Gastric phase	Intestinal phase	Sample	Salivary phase	Gastric phase	Intestinal phase
S	(107.72±1.67)^{aA}	(108.98±6.04)^{aA}	(103.81±3.51)^{aA}	S	(100.26±5.48)^{aA}	(104.56±4.07)^{aA}	(96.50±2.86)^{aA}
S + 20 % L	(100.78±0.03)^{bA}	(101.59±0.48)^{aA}	(100.80±8.41)^{aA}	S + 20 % L	(96.75±2.10)^{aA}	(89.47±3.20)^{aAB}	86.19±3.85)^{aB}
Mean	(104.25±4.90)^{A}	(105.28±5.23)^{A}	(102.30±2.12)^{A}	Mean	(98.51±2.48)^{A}	(97.01±10.67)^{A}	(91.34±7.129)^{A}
FA bioaccessibility/%				Q bioaccessibility/%			
Sample	Salivary phase	Gastric phase	Intestinal phase	Sample	Salivary phase	Gastric phase	Intestinal phase
S	(101.16±4.11)^{aA}	(97.67±5.70)^{aA}	(92.50±0.43)^{aA}	S	(101.46±1.01)^{aA}	(99.86±0.82)^{aAB}	(98.06±0.02)^{bB}
S +20 % L	(108.83±5.47)^{aA}	(98.22±10.95)^{aA}	(93.81±3.85)^{aA}	S + 20 % L	(101.21±0.54)^{aA}	(98.82±0.93)^{aAB}	(100.11±0.11)^{aB}
Mean	(105.00±5.42)^{A}	(97.95±0.39)^{AB}	(93.15±0.92)^{B}	Mean	(101.33±0.18)^{A}	(99.34±0.73)^{B}	(99.08±1.45)^{B}

Data are presented as mean value±S.D, N=2-5**.** Different small letters indicate significant difference at p≤0.05 for each phase separately. Capital letters indicate significant difference at p≤0.05 between all phases together. S=spinach, L=lemon juice, TP=total polyphenols, TF=total flavonoids, TPA=total phenolic acids, OA=oxalic acid, *L*-AA=*L*-ascorbic acid, *p*-CA=*p*-coumaric acid, FA=ferulic acid, Q=quercetin.

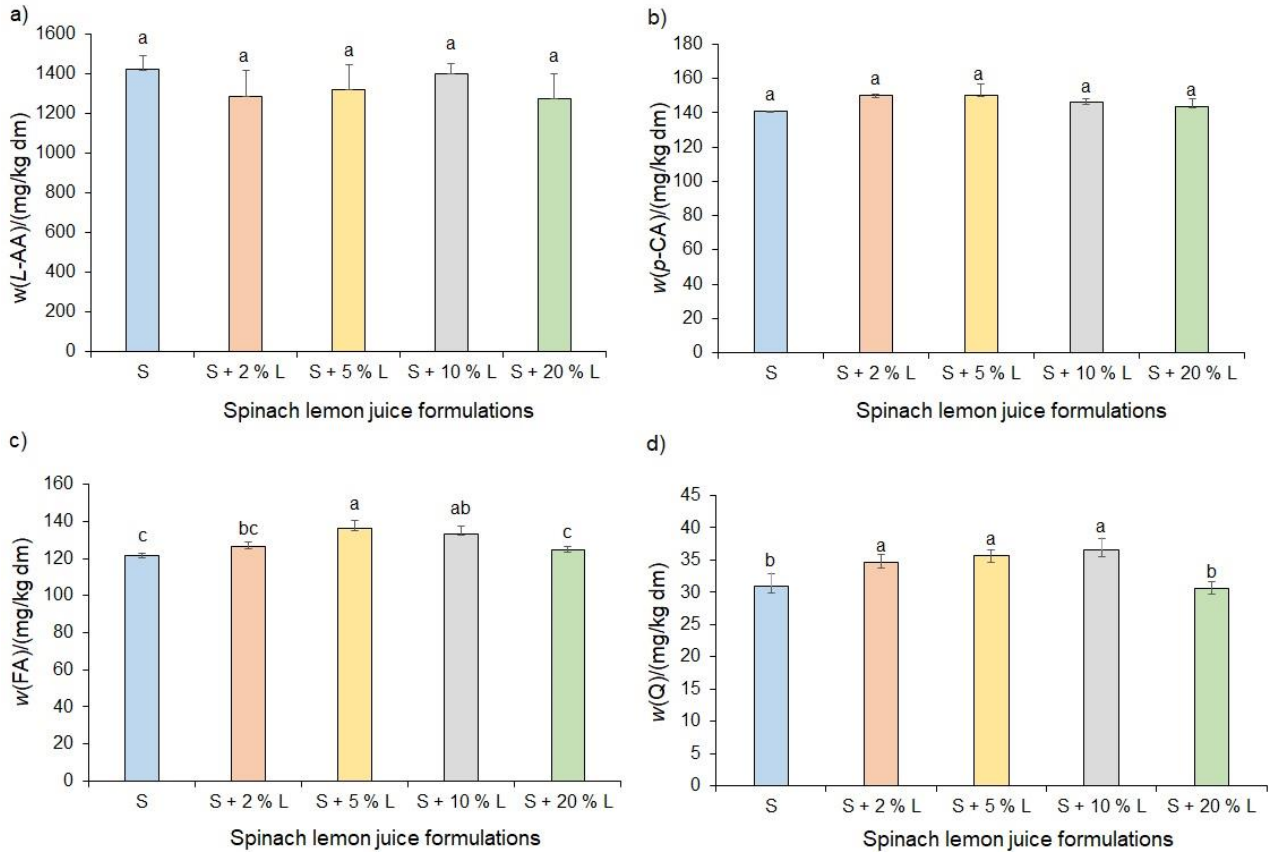

Fig. 1. RP-HPLC analysis of: a) *L*-ascorbic acid=*L*-AA and individual phenolic compounds b) *p*-coumaric acid=*p*-CA, c) ferulic acid=FA and d) quercetin=Q in predigested (original sample) spinach=S lemon juice=L formulations (0, 2, 5, 10 and 20 %). Data are presented as mean value±S.D, N=3. Different letters indicate significant difference at p≤0.05

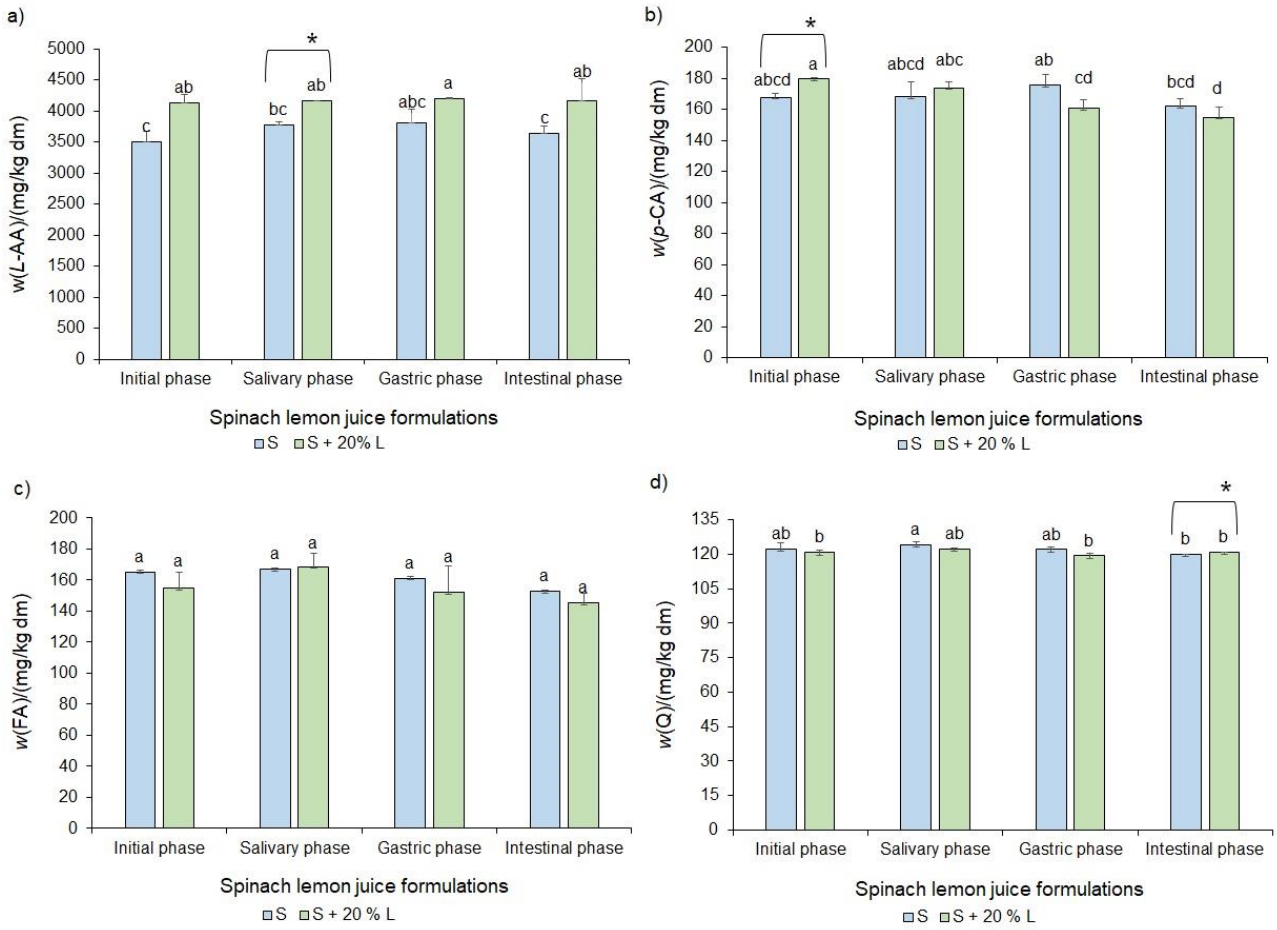

Fig. 2. RP-HPLC analysis of: a) *L*-ascorbic acid=*L*-AA and individual phenolic compounds b) *p*-coumaric acid=*p*-CA, c) ferulic acid=FA and d) quercetin=Q in digested (initial phase, salivary phase, gastric phase and intestinal phase) spinach=S lemon juice=L formulations (0 and 20 %). Data are presented as mean value±S.D, N=3. Different letters indicate significant difference at p≤0.05 for all samples and phases together. Aster indicate significant difference at p≤0.05 for each phase individually

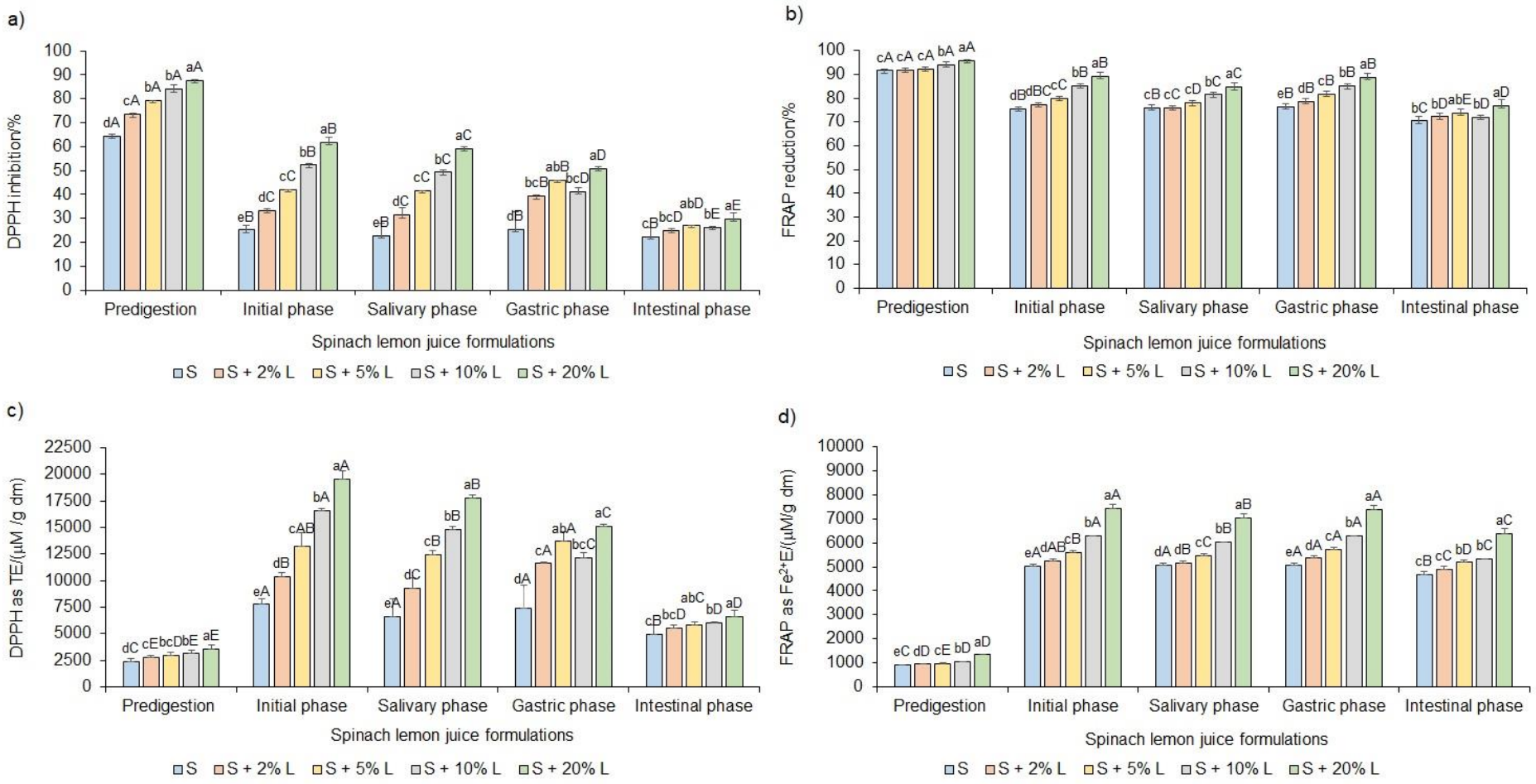

Fig. 3. Antioxidant activity: a) DPPH inhibition/% and c) DPPH as TE/(μM/g dm), b) FRAP reduction/% and d) FRAP as Fe^{2+}E/(μMg dm) in predigested and digested (initial phase, salivary phase, gastric phase and intestinal phase) spinach lemon juice formulations (0, 2, 5, 10 and 20 %). Data are presented as mean value±S.D, N=4 Different small letters indicate significant difference at p≤0.05 for each phase separately. Capital letters indicate

significant difference at $p \leq 0.05$ between all phases together. S=spinach, L=lemon juice, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = ferric ion reducing antioxidant power

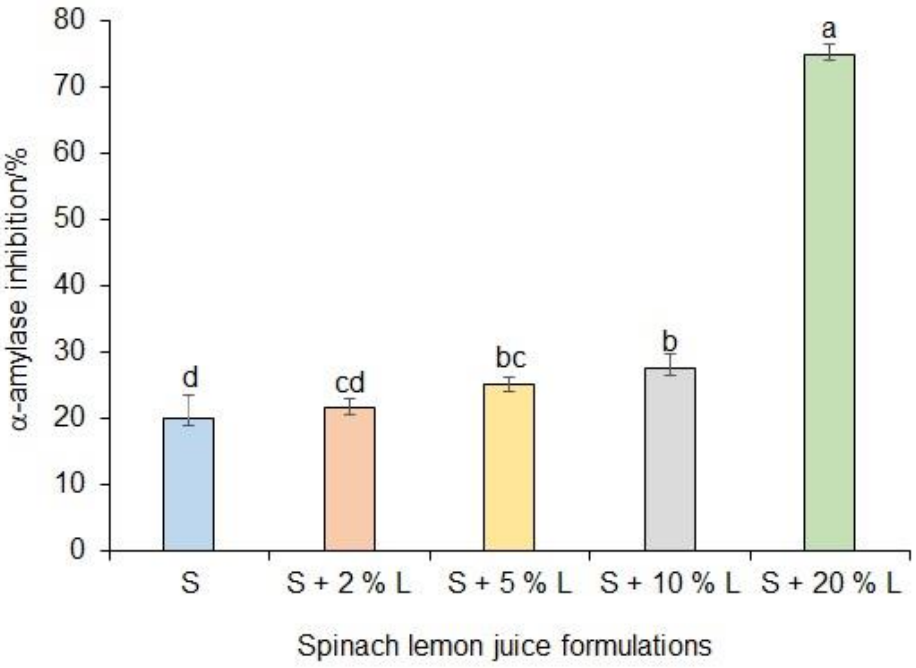

Fig. 4. α-amylase enzyme inhibitory activity of predigested spinach=S lemon juice=L formulations (0, 2, 5, 10 and 20 %). Data are presented as mean value±S.D, N=4. Different letters indicate significant difference at $p \leq 0.05$

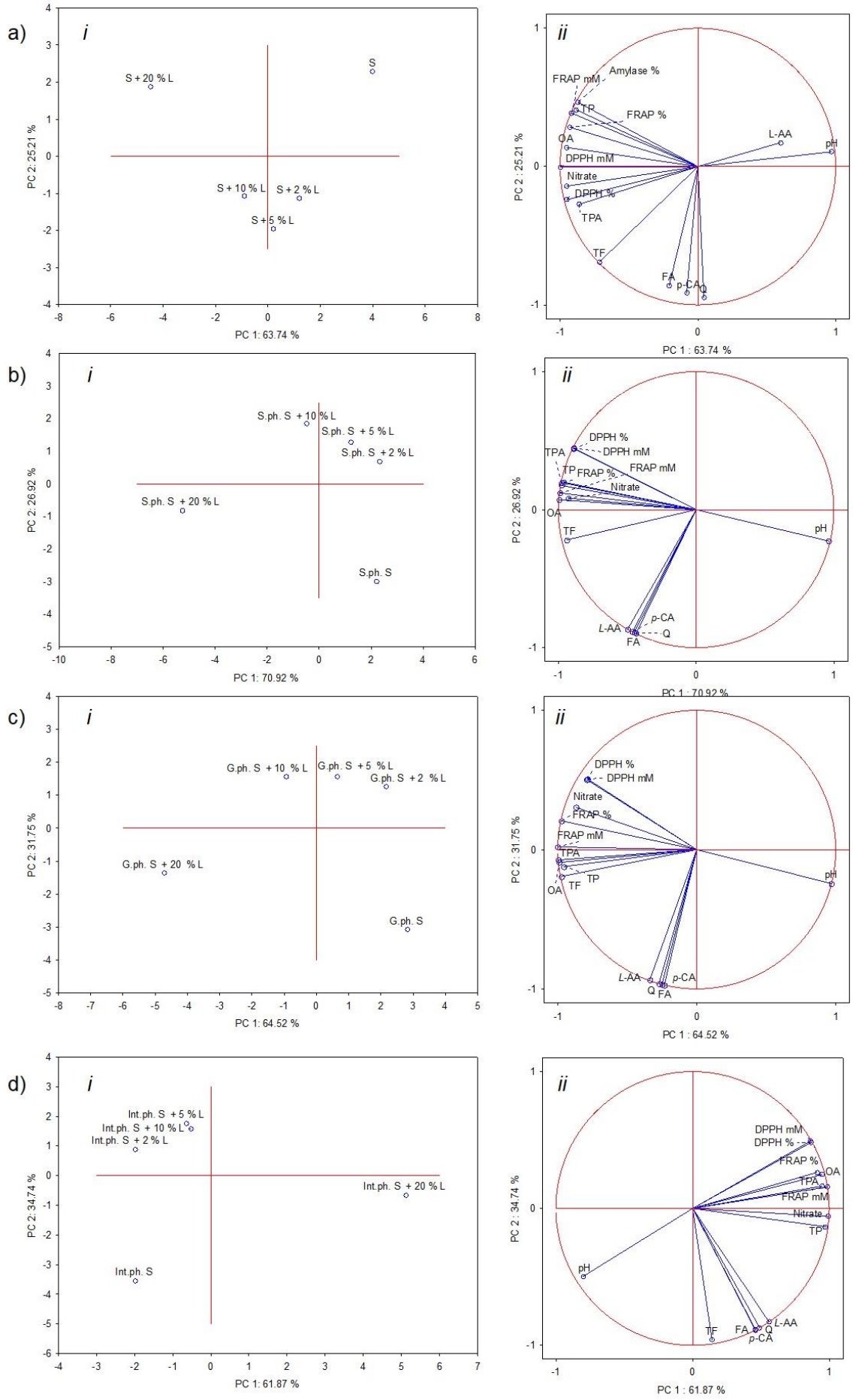

Fig. 5. The principal component analysis (PCA) performed on the correlation matrix of average values of pH, TP=total phenols, TF=total flavonoids, TPA=total phenolic acids and individual phenolic compounds (*p*-CA=*p*-coumaric acid, FA=ferulic acid and Q=quercetin), *L*-AA=*L*-ascorbic acid, OA=oxalic acid and nitrate, antioxidant activity (DPPH inhibition % and DPPH as TE/(mM/g dm), FRAP reduction/% and FRAP as Fe^{2+}E/(mM/g dm) and antidiabetic (α-amylase inhibition/%) activity in a) predigested and digested b) S.ph.=salivary phase, c) G.ph.=gastric phase and d) Int.ph.=intestinal phase spinach=S lemon juice=L formulations (0, 2, 5, 10 and 20 %). (i) Score plot separating the spinach lemon formulation, and (ii) the loading plot of pH, metabolites and antioxidant and antidiabetic activity as variables

Table S1. pH values of spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

	Original sample pH	Initial phase pH	Salivary phase pH	Gastric phase pH	Intestinal phase pH
S	(5.06±0.05)^{aD}	(6.66±0.04)^{aC}	(6.82±0.07)^{aB}	(2.24±0.01)^{aE}	(7.04±0.02)^{aA}
Š + 2 % L	(4.65±0.01)^{bD}	(6.60±0.02)^{bC}	(6.65±0.01)^{bB}	(2.10±0.02)^{bE}	(6.70±0.01)^{bA}
Š + 5 % L	(4.04±0.02)^{cD}	(6.13±0.03)^{cC}	(6.16±0.02)^{cB}	(2.04±0.01)^{cE}	(6.64±0.01)^{cA}
Š + 10 % L	(3.83±0.01)^{dD}	(5.29±0.02)^{dC}	(5.28±0.02)^{dB}	(1.94±0.01)^{dE}	(6.30±0.01)^{dA}
Š + 20 % L	(3.34±0.01)^{eC}	(4.45±0.03)^{eB}	(4.39±0.01)^{eB}	(1.78±0.02)^{eD}	(6.12±0.02)^{eA}

Data are presented as mean value±S.D, N=4. Different letters indicate significant difference at p≤0.05 for each phase separately. Capital letters indicate significant difference at p≤0.05 between all phases together. S=spinach, L=lemon juice.

Table S2. Pearson's correlation coefficient between spectrophotometric determination of total polyphenols, oxalic acid and nitrate, antioxidant activity and pH in digested (salivary phase, gastric phase and intestinal phase) spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

Salivary phase	TP	TF	TPA	OA	Nitrate	DPPH %	DPPH mM	FRAP %	FRAP mM	pH
TP	1.000									
TF	0.502	1.000								
TPA	**0.875**	0.567	1.000							
OA	**0.788**	0.615	**0.943**	1.000						
Nitrate	**0.809**	0.406	**0.833**	**0.766**	1.000					
DPPH %	**0.726**	0.418	**0.922**	**0.935**	**0.718**	1.000				
DPPH mM	**0.725**	0.416	**0.922**	**0.934**	**0.718**	**1.000**	1.000			
FRAP %	**0.850**	0.590	**0.967**	**0.901**	**0.731**	**0.868**	**0.868**	1.000		
FRAP mM	**0.829**	0.609	**0.975**	**0.986**	**0.769**	**0.940**	**0.939**	**0.961**	1.000	
pH	**-0.748**	-0.560	**-0.946**	**-0.966**	**-0.714**	**-0.976**	**-0.973**	**-0.932**	**-0.980**	1.000
Gastric phase	TP	TF	TPA	OA	Nitrate	DPPH %	DPPH mM	FRAP %	FRAP mM	pH
TP	1.000									
TF	**0.795**	1.000								
TPA	**0.735**	**0.906**	1.000							
OA	**0.840**	**0.875**	**0.938**	1.000						
Nitrate	**0.738**	0.448	0.570	**0.770**	1.000					
DPPH %	**0.640**	**0.758**	**0.658**	**0.747**	0.614	1.000				
DPPH mM	**0.634**	**0.753**	**0.651**	**0.740**	0.609	**1.000**	1.000			
FRAP %	**0.710**	**0.781**	**0.897**	**0.927**	**0.750**	**0.817**	**0.812**	1.000		
FRAP mM	**0.801**	**0.858**	**0.941**	**0.988**	**0.769**	**0.792**	**0.786**	**0.974**	1.000	
pH	**-0.788**	**-0.773**	**-0.822**	**-0.940**	**-0.836**	**-0.867**	**-0.863**	**-0.959**	**-0.964**	1.000
Intestinal phase	TP	TF	TPA	OA	Nitrate	DPPH %	DPPH mM	FRAP %	FRAP mM	pH
TP	1.000									
TF	0.350	1.000								
TPA	**0.757**	0.277	1.000							
OA	0.593	0.115	**0.964**	1.000						
Nitrate	**0.761**	0.427	**0.867**	**0.792**	1.000					
DPPH %	0.623	0.262	**0.896**	**0.849**	**0.856**	1.000				
DPPH mM	0.620	0.262	**0.890**	**0.841**	**0.854**	**1.000**	1.000			
FRAP %	**0.857**	0.047	**0.815**	**0.719**	0.652	**0.673**	**0.669**	1.000		
FRAP mM	**0.742**	0.191	**0.993**	**0.969**	**0.828**	**0.876**	**0.870**	**0.853**	1.000	
pH	-0.477	0.151	**-0.714**	**-0.747**	-0.653	**-0.902**	**-0.904**	-0.634	**-0.756**	1.000

Bold values are significant at p≤0.05. TP=total polyphenols, TF=total flavonoids, TPA=total phenolic acids, OA=oxalic acid, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = ferric ion reducing antioxidant power.

Table S3. Pearson's correlation coefficient between spectrophotometric determination of total polyphenols, oxalic acid and nitrate, antioxidant activity, antidiabetic activity and pH in predigested spinach lemon juice formulations (0, 2, 5, 10 and 20 %)

Predigested sample	TP	TF	TPA	OA	Nitrate	DPPH %	DPPH	FRAP %	FRAP	α-amylase	pH
TP	1.000										
TF	0.233	1.000									
TPA	0.616	0.524	1.000								
OA	**0.670**	0.377	**0.823**	1.000							
Nitrate	0.440	**0.729**	0.666	**0.693**	1.000						
DPPH %	0.584	**0.817**	**0.756**	**0.806**	**0.925**	1.000					
DPPH	**0.685**	**0.723**	**0.836**	**0.788**	**0.900**	**0.950**	1.000				
FRAP %	**0.890**	0.319	0.648	**0.835**	**0.667**	**0.748**	**0.766**	1.000			
FRAP	**0.850**	0.279	**0.705**	**0.786**	**0.705**	**0.725**	**0.848**	**0.905**	1.000		
α-amylase	**0.833**	0.076	**0.712**	**0.730**	0.529	0.548	**0.725**	**0.834**	**0.959**	1.000	
pH	-0.652	**-0.780**	**-0.747**	**-0.738**	**-0.929**	**-0.970**	**-0.978**	**-0.780**	**-0.811**	-0.651	1.000

Bold values are significant at p≤0.05. TP=total polyphenols, TF=total flavonoids, TPA=total phenolic acids, OA=oxalic acid, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = ferric ion reducing antioxidant power.