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Manuscript Draft

Manuscript Number: JFF-D-18-00599R1

Title: Stinging nettle (Urtica dioica L.) as a functional food additive in egg pasta: enrichment and bioaccessibility of Lutein and beta-Carotene

Article Type: Full Length Article

Keywords: lutein; b-carotene; stinging nettle; carotenoid-enriched food; bioaccessibility; HPLC-UV/Vis-APCI-MS/MS

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and after 48 hours of colonic fermentation. Nettle capsules release carotenoids with a maximum bioaccessibility at 24 hours of colonic fermentation, similarly to non-enriched egg pasta. Nettle enriched egg pasta shows the highest levels for bioaccessibility at a lower colonic fermentation time (i.e., 2 hours).

*Cover Letter

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Prof. Elvira de Mejia
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Dear Associate Editor,

I am sending you a revised copy of the Manuscript JFF-D-18-00599 entitled *Stinging nettle (Urtica dioica L.) as a functional food additive in egg pasta: enrichment and bioaccessibility of Lutein and β-Carotene* to address the Reviewers' comments.

Appended to this letter is our response (in *italic*) to comments raised by Referees. All requests were addressed and I hope that additions I have made resolve your concerns about the Manuscript. As requested, a revised manuscript version with highlighted changes in red was prepared. We hope that the Manuscript can now be accepted for publication on the Journal of Functional Foods.

Thank you once again for your time and interest.

Sincerely,

Nicola Marchetti, PhD

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Reviewer #1

- The presented manuscript is enough interesting and novel, but their major shortcoming is lack of statistical analysis (see tables and figures). Statistical analysis of the results should be carried out. This is a prerequisite for publication.
  The authors accept this remark and we included statistical analysis in tables 1 and 2, and figures 2 and 3. Experimental raw data sets were subjected to the analysis of variance (one-way ANOVA) and a Tukey's multiple comparisons adjustment to test for significant differences between the means. P-values < 0.05 were regarded as significant. Accordingly with statistical analysis data are marked with a, b, c and d letters in apex after each numerical value. Values that were statistically similar were marked with identical letters in apex ($P > 0.05$). In figure 2 and 3 letters with analogous meaning are placed above histogram bars.
  Subsection 2.9, named "Statistical Analysis", was added to the Manuscript. Also, captions for tables 1 and 2, and for figures 2 and 3 were modified accordingly.

Reviewer #2

- In my opinion the manuscript is well written and the experimental plan is well presented. The use of stinging nettles as an ingredient in egg pasta has been evaluated with respect to food enrichment with carotenoids. Bioaccessibility of lutein and b-carotene have been estimated by dynamic simulation of the digestion process providing results which could be interesting for researchers in the field. Therefore I believe that the manuscript could be published in the Journal of Functional Food.
  The authors thank Reviewer #2.

Editor

- Statistical analysis in tables and figures MUST be included in order to be eligible to publish this manuscript.
  The authors accept Editor's request and statistical analysis was added as described in the answer to Reviewer #1 and also reported in the manuscript.

Highlights

- Stinging nettle leaves were evaluated as a source of carotenoids for food enrichment.
- Enrichment of fresh egg pasta with nettle dried leaves was studied.
- A dynamic gastro-intestinal model was employed.
- Bioaccessibility of lutein and β-carotene was investigated.

Stinging nettle (*Urtica dioica L.*) as a functional food additive in egg pasta: enrichment and bioaccessibility of Lutein and β-Carotene

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Abstract

The use of stinging nettles as an ingredient in egg pasta has been evaluated with respect to food enrichment with carotenoids. Bioaccessibility of lutein and β-carotene has been estimated by dynamic simulation of the digestion process, with particular attention to duodenum and colon stages. Higher bioaccessibility for the two carotenoids occurs between 2 and 24 hours of colonic fermentation and it is around 35% for lutein and 10% for β-carotene. However, the results reveal that the food matrix has a significant role in carotenoid release during the digestion process. In general, nettle enriched pasta has a lower carotenoid bioaccessibility than dietary supplement at duodenum and after 48 hours of colonic fermentation. Nettle capsules release carotenoids with a maximum bioaccessibility at 24 hours of colonic fermentation, similarly to non-enriched egg pasta. Nettle enriched egg pasta shows the highest levels for bioaccessibility at a lower colonic fermentation time (i.e., 2 hours).

Keywords: lutein, β-carotene, stinging nettle, carotenoid-enriched food, bioaccessibility, HPLC-UV/Vis-APCI-MS/MS

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1. Introduction

According to the Leatherhead Food Research Company (Leatherhead, 2016) the global trend for the market of functional foods increased by about 31.5% from 2007 to 2011. Japan (+36.8%), Australia (+32.3%) and Europe (+30.4%) are the the leading regions for this trend. Furthermore, Mintel Agency forecasted that the number of functional new products launched on the global market would reach a value of about 5,500 in 2011 and their market is expected to grow 25% by 2017 (Mintel, 2016). The Institute of Food Technologists (IFT, 2016) published a report compiled by a panel of experts which outlines the reasons for the rapid increase of scientific publications involving functional foods from different viewpoints. Functional foods and molecular nutrition can give us a better understanding of: (i) action mechanisms for known nutrients; (ii) their dose-response relationships; and, (iii) clinical outcomes and individual variation in response. The main goal is to achieve deeper nutraceutical knowledge and to develop better functional foods. On the one hand, many fruits, vegetables, edible plants and herbs, and processed and traditional foods have been characterized in terms of their nutritional components with advanced analytical techniques. On the other hand, scientists are constantly pursuing a precise comprehension of the potential roles of phytochemicals in health. In addition, epidemiological studies have studied health status and diet. Clinical evidence has shown the nutrition outcomes on cells, tissues or organs (i.e., new and existing biomarkers). In-vitro and in-vivo studies have demonstrated that bioactive compounds can interfere with biochemical processes at a molecular level and then influence the development of chronic diseases.

Chemopreventive effects of bioactive compounds in fresh or processed food are currently the object of investigation. Several different scientific communities are pursuing the collection of more accurate data to improve the possible beneficial health effects of conscious nutrition and the consumption of functional foods. This requires a strong collaborative research effort between medicine, nutrition, biochemistry, food chemistry and food technology.

This study aims to apply the nutraceutics contained in wild, edible plants, such as stinging nettle (*Urtica dioica L.*), as an ingredient for new functional foods of common use, such as pasta. In this study, egg pasta enriched with nettle dried leaves is used to increase the

carotenoids content and to obtain a more effective functional food. In addition, effect of ingredients, cooking process, food matrix and shape on bioaccessibility of carotenoids are investigated: stability of functional ingredients and relative amount released from the food matrix into gastric and intestinal fluids during digestion can be established (Bergantin et al., 2017). Characterization of these digestion fluids is crucial since the bioaccessible fraction is then subjected to absorption processes that determine ingredient bioactivity (i.e., health effects). Nettle leaves and roots have been used since ancient times as a medicinal remedy. The edible fresh or dried parts of nettle plants have been extensively used in food preparation (Kavalali, 2003; Sansanelli & Tassoni, 2014). In a previous work (Bonetti et al., 2016), the polyphenols in nettle leaves were quantitatively determined, and their bioaccessibility and bioavailability from the same enriched foods were investigated after in an in-vitro digestion simulated process. Here, the addition of nettle to processed food was investigated with attention to major carotenoids. Dried nettle leaves was studied because this is the most commonly employed additive in fresh pasta processing in food industry. All pasta types considered in this work were produced by a local firm (Pastificio Andalini S.p.A, http://www.andalini.it/en/) that already released this nettle enriched egg pasta on the market. The amount of each ingredient refers to the recipes previously developed and employed by this food industry. No investigation of the influence of pasta drying process, storage conditions or shelf life on the carotenoid content was made.

Enrichment of pasta with functional ingredients as well as addition of vegetables for sensory reason is not new. These aspects have been extensively studied from a technological and nutritional point of view. It was mainly due to modification of rheological properties of pasta after addition of non conventional ingredients: it is known that enrichment with vegetable and herbs can cause weakening of gluten network and negatively influence with starch gelatinization. These changes might modify both sensorial and health-promoting characteristics of final pasta. Change of color upon enrichment is a positive aspect, while change of flavor has to be carefully considered, basically due to the “green” taste given by added vegetables, for example. This latter point might not meet consumers’ taste and, in this light, the amount of added ingredients have to be evaluated. In reference to health-promoting characteristic of enriched pasta, it is well known that a negative aspect of vegetable pasta is the potential increase of the

glycaemic index. This typically arises from a modification of protein network upon partial replacement of flour fraction with vegetable matrix and consequent increased exposure of starch granules. Under these conditions water absorption is promoted, starch granules swell and glycaemic index raises due to augmented starch bioavailability (Oliviero & Fogliano, 2016; van Boekel et al., 2010).

Carotenoids are fat soluble plant pigments that are also present in algae, fungi and bacteria. Over 600 different carotenoids have been identified and some are very important to human health thanks to their antioxidant properties. Dietary carotenoids, such as carotene, lutein, zeaxanthin, cryptoxanthin and lycopene, are involved in scavenging of free radicals and elimination of peroxides with consequent effect of protecting cells from damage and oxidative stress. Carotenoids can also enhance the immune function by stimulating lymphocytes production, increased activity of neutrophils and macrophages, and production of cancer immunity (Mortensen, Skibsted, Sampson, Rice-Evans, & Everett, 1997). Other possible chemopreventive effects of carotenoids, such as against cardiovascular diseases, are primarily based on their antioxidant characteristics. The inherent lipophilicity of these compounds has limited their potential applications as hydrophilic additives without significant formulation efforts (Lockwood, O'Malley, & Mosher, 2003). The lipid content of foods or lipidic transporter particles increase the adsorption of carotenoids. Hence, delivery of phytonutrients, as well as the formulation of processed food and synergic effects between different food matrices have to be investigated in depth. Results can produce tangible fallouts, such as the improvements of functional and enriched foods, health and nutritional benefits. Recent trends in phytochemical studies and their relation with health beneficial effects have revealed that it is crucial to gain a clear understanding of the mechanisms involved in chemoprevention from phytochemical intake (D'Archivio, Filesi, Varí, Scazzocchio, & Masella, 2010).

2. Materials and Methods

2.1. Chemicals

Potassium chloride (KCl), potassium thiocyanate (KSCN), sodium dihydrogen phosphate (NaH_2PO_4), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium hydrogen carbonate

($NaHCO_3$), urea ($CO(NH_2)_2$), hydrochloric acid (HCl), sodium hydroxide (NaOH), formic acid (HCOOH), phosphate buffer (PBS, pH=7.5), α-amylase (930 U/mg), pepsin A (674 U/mg), pancreatin (762 U/mg), bile salts (B8631), β-carotene ($\geq$ 97%), and lutein ($\geq$ 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (HPLC and LC-MS grade) were also sourced from Sigma-Aldrich. Ultra pure water was obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

An Ultraturrax (model T18 basic) was purchased from IKA (Staufen im Breisgau, Germany). A refrigerated multi-speed centrifuge (model 5810R) was purchased from Eppendorf (Hamburg, Germany). A 5 L bioreactor for in vitro dynamic digestion studies was obtained from Infors (Bottmingen, Switzerland). A Stomacher unit was purchased by IUL Instrument (Barcelona, Spain). The HPLC system was a Surveyor Plus (Thermo Scientific, Waltham, MA, USA) equipped with solvent delivery system, degasser, quaternary micro pump (4 channels), thermostated autosampler and column compartment. The MS detector was a LTQ-XL linear ion trap (Thermo Scientific, Waltham, MA, USA). The UV-vis detector was a diode array detector, model G1315C with a 5μL semi-micro flow cell (Agilent, Palo Alto, CA, USA).

2.3. Reagent Preparation

Electrolyte stock solutions were prepared at the following concentrations: KCl 89.6 g/L; KSCN 20g/L; NaH_2PO_4 88.8 g/L; $NaHCO_3$ 84.7 g/L; NaCl 175.3 g/L; Na_2SO_4 57 g/L; $CO(NH_2)_2$ 25 g/L. These solutions were used to employ simulated fluids with the gastro-intestinal model to simulate digestion. Three different fluids were prepared through a modified procedure, as reported by Minekus (Minekus, 2015; Minekus et al., 2014).

Simulated salivary fluid (SSF): 10 mL of KCl; 10 mL of KSCN; 10 mL of NaH_2PO_4; 10 mL of Na_2SO_4; 1.7 mL of NaCl; 20 mL $NaHCO_3$; 8 mL of $CO(NH_2)_2$; 290 mg of α-amylase.

Simulated gastric fluid (SGF): 25 mL of HCl 0.1 N; 5 g of pepsin.

Simulated intestinal fluid (SIF): 200 mL of water; 25 mL of pancreatin (8 mg/mL); 25 mL of bile salts (50 mg/mL); pH was adjusted to 6.8 with $NaHCO_3$ 0.5 N.

All of the enzyme solutions were freshly prepared, preincubated at 37°C before use and stored at 4°C for maximum three days. Enzymes provided by the supplier were assayed according to reference tests, as reported in literature (Minekus et al., 2014) and manufacturer's protocols: (i) α-amylase assay was based on spectrophotometric stop reaction using soluble potato starch as a substrate; (ii) pepsin activity assay was based on spectrophotometric stop reaction using hemoglobin as substrate; (iii) pancreatin activity was assayed in terms of its trypsin and chimotrypsin activity based on continuous spectrophotometric rate determination using p-toluene-sulfonyl-L-arginine methyl ester and N-benzoyl-L-tyrosine ethyl ester as substrates, respectively.

2.4. Samples

Stinging nettle plants were cultivated and major carotenoids were determined in fresh leaves during four consecutive growth stages: vegetative (UD-V); flowering (UD-F); seed maturation (UD-S); and, quiescence (UD-Q). Two different shapes of egg pasta were considered: curly, short, thick (P1); straight, long, thin (P2). P1-T and P2-T samples refer to traditional pasta without nettle as an ingredient. P1-O and P2-O samples contain nettle enriched egg pasta. In addition to water, the main ingredients of P1 and P2 were durum wheat semolina and 24% (w/w) egg. The nettle enriched pasta contains 2.5% (w/w) dried nettle leaves. The carotenoids' stability was evaluated by comparing the amount before and after cooking, while bioaccessibility experiments were undertaken starting from boiled pasta. Enriched pasta samples were studied and compared with a commercial nettle dietary supplement as a reference nettle rich food (CAP). Finally, 40 g of cooked pasta and two capsules were digested with the in-vitro dynamic gastro-intestinal model for bioaccessibility studies.

2.5. Carotenoids extraction

The carotenoids were extracted from the pasta (raw and cooked) and supplement capsules using a modified literature procedure from Panfili et al. (Panfili, Fratianni, & Irano, 2004). About 40 g of pasta are hydrolyzed in a centrifuge tube (50 mL) by adding 5 mL of Pyrogallol 60 g/L solution in Ethanol, 2 mL of Ethanol, 2 mL of NaCl 10 g/L aqueous solution and 2 mL of KOH

600 g/L aqueous solution. The sample is then homogenized with Ultraturrax for 3 min and it is then gently shaken for 45 min at 70°C. The tube is cooled in an ice bath for 5 min and then 15 mL of NaCl (10 g/L) are added. The sample is extracted twice with 15 mL Hexane:Ethyl-Acetate 9:1 + BHT 0.1% w/v.The collected organic layers are evaporated at 30°C using a rotary evaporator. The dried extract is resuspended with mobile phase for HPLC analysis and filtered (nylon, 0.22 μm syringe filter) before injection.

2.6. HPLC-UV/Vis-APCI-MS/MS Analysis

Chromatographic separations were performed on a Kinetex (Phenomenex) 100 x 2.1 mm column packed with 2.6 μm particles and under the following conditions: pure water, ACN and THF as mobile phases; flow rate 120 μL/min; and, column temperature 40°C. The gradient elution conditions were: 0-12 min 30%-5% water and 20%-45% THF (ACN constant at 50%), 12-20 min 50%-20% ACN and 45%-75% THF (water constant at 5%).

An external calibration method was applied for quantitative purposes. The calibration ranges were shifted by one order of magnitude between Lutein and β-carotene according with carotenoid abundance in the extracts: it was 0.05-2 μg/mL for lutein and 0.005-0.2 μg/mL for β-carotene. Linear regression for both sets provided correlation coefficients higher than 0.998. The calibration curve for β-carotene showed a slightly higher sensitivity than that for lutein (slope of calibration curve is 15% larger for β-carotene). This represented an important advantage because β-carotene is less abundant in these samples than lutein and, hence, can be more accurately detected.

2.7. Bacterial strains and growth conditions

To mimic the physiological conditions of the colonic intestinal tract, 13 commercial probiotic bacterial strains were used, namely: *Lactobacillus animalis* CECT 4060T, *Lactobacillus casei* CECT 4180, *Lactobacillus casei rhamnosus* CECT 278T, *Lactobacillus plantarum* CECT 220, *Lactobacillus rhuminis* CECT 4061T, *Lactobacillus casei casei* CECT 277, *Bifidobacterium breve* CECT 4839T, *Bifidobacterium adolescentes* CECT 5781T, *Bifidobacterium bifidum* CECT 870T, *Corinebacterium vitaeruminis* CECT 537, *Streptococcus fecalis* CECT 407, *Eubacterium crispa-*

tus CECT 4840, *Saccharomyces cerevisiae* CECT 1324, which were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) and delivered in sterile 18% glycerol. For longer survival and higher quantitative retrieval of the cultures, they were stored at -80°C. When needed, recovery of the strains was undertaken by two consecutive subcultures in an appropriate media before use (Meca, Manes, Font, & Ruiz, 2012).

2.8. In-vitro dynamic digestion model

The in-vitro dynamic gastro-intestinal model consisted of a reactor, representing the stomach, the small intestine (duodenum), the ascending colon, the transverse colon, and the descending colon interconnected by plastic tubes and peristaltic pumps, as previously described by Ferreri et al. (Ferrer, Manyes, Manes, & Meca, 2015). The unit was fully computer-controlled (LabVIEW software) for the addition of: (a) food to the stomach, (b) buffers to adjust pH of all compartments, and (c) pancreatic juice to the small intestine. The transit time of the flow of the intestinal content between reactors was also automatically computer-controlled. The reactors was double glass-jacketed to keep the inner part thermostated at 37 °C with water. The pH was automatically controlled by addition of HCl 0.2 M to the stomach vessel and NaOH 0.5 M to the small intestine vessel to keep pH values of 2.0 and 6.5, respectively.

Salivar phase—The samples that were prepared as previously described (40 g of cooked pasta or two supplement capsules) were mixed with 60 mL of SSF and mixed in a plastic bag that contains 1 L of water at 37 °C for 30 sec by a Stomacher homogenizer. This was then introduced into the fermenter vessel for gastric digestion.

Gastric phase—The bolus from the previous phase is introduced into the fermenter vessel together with 25 mL of SGF. The pH was adjusted to 2 and the bioreactor was kept under mild agitation at 37°C for 2 h.

Intestinal phase—Small intestine digestion was simulated by increasing the pH to 6.8 with $NaHCO_3$ 0.5 N. Thereafter, SIF was introduced and incubation was done under mild agitation at 37°C for 2 h. An aliquot of duodenal fluid was sampled and centrifuged at 2,500 g for 5 min at 4°C. Then, the supernatant was recovered and free carotenoids were extracted as reported above, before the sample was injected for the analysis. To simulate colonic fermentation, the

microbial strains were inoculated in a fermenter vessel at 10^{14} CFU/mL and incubated at 37°C for a maximum of 48 hours. After 2, 24 and 48 hours, the aliquots of the mixture were sampled and centrifuged at 10,000 g for 5 min at 4°C. The supernatants were withdrawn, the free carotenoids were extracted, and the samples were analyzed.

In-vitro bioaccessibility can be estimated at laboratory scale using chemical extraction of carotenoids from reference food in solution and under experimental conditions that mimic the mixing and processing of GI fluids. Total recoverable lutein and β-carotene is firstly determined in nettle and in egg pasta, then bioaccessibility is calculated as reported in Eq.1:

$$\text{Bioaccessibility\%} = \frac{\mu g/g \text{ of Lutein extracted in GI fluids}}{\mu g/g \text{ of total recoverable Lutein}} \times 100 \quad (1)$$

and analogously for β-carotene.

2.9. Statistical Analysis

All experimental data sets were subjected to the analysis of variance (one-way ANOVA) and a Tukey's multiple comparisons adjustment to test for significant differences between the means. P-values < 0.05 were regarded as significant. Experimental data for lutein and β-carotene can belong to the same or different groups and this was indicated by means of *a*, *b*, *c* and *d* letters in apex after each numerical value (see tables 1 and 2): data marked with identical letters refer to statistically similar values (P > 0.05). Letters were also added with the same meaning above histogram bars in figure 2 and 3 where bioaccessibility for different types of pasta and food supplement were compared for each digestion phase.

3. Results and Discussion

3.1. Carotenoid determination by HPLC-UV/Vis-APCI-MS/MS

HPLC-UV/Vis analysis of the sample extracts (see Fig.1) revealed six main resolved peaks: one peak at high intensity (peak 4, t_r = 9.87 min), three peaks at medium intensity (peak 2, t_r = 6.06 min; peak 3, t_r = 6.85 min; peak 5, t_r = 11.27 min) and two at low intensity (peak 1, t_r = 5.65 min and peak 6, t_r = 19.65 min). The chromatogram reported in Fig.1 was recorded at λ=450

nm. Among these there were small, poorly resolved peaks between 7.50 and 9.00 min. Some of these peaks were tentatively identified by coupling in series a mass spectrometric detector to the previous system (HPLC-UV/Vis-APCI-MS/MS).

Compound 4 was tentatively identified as lutein on the basis of the MS/MS spectrum (see Fig. S1, Supporting Information): characteristic fragments from CID transitions of $[M+H]^+$=569 *m/z* protonated molecule, such as 430, 477, 533 and 551 *m/z*, are found. A total of 551 and 533 *m/z* fragments correspond to dehydrated product ions yielded by the loss of one and two water molecules (i.e., [M+H-nH_2O]), respectively (Crupi, Milella, & Antonacci, 2010). This type of transition has been widely described in the literature for hydroxylated carotenoids (i.e., xanthophylls) (Rivera, Christou, & Canela-Garayoa, 2014). Confirmation was also achieved by comparison of chromatographic retention time and MS/MS spectrum with lutein analytical standard. Compound 6 referred to $[M+H]^+$=537 *m/z* protonated molecule and its MS/MS spectrum contained typical fragment ions that are amenable to β-carotene, such as 177, 269, 399, 413, 444, and 457 *m/z* (see Fig. S2, Supporting Information). 537→457 *m/z* and 537→444 *m/z* transitions addressed for methyl-cyclopentadiene (i.e., $[M+H-80]^+$) and toluene ($[M+H-92]^+$) loss, respectively. Further transitions, 537→413 *m/z* and 537→399 *m/z*, take into account for loss of β-ionone moiety (i.e., $[M+H-124]^+$) and methylated β-ring (i.e., $[M+H-137]^+$), respectively (Rivera, Christou, & Canela-Garayoa, 2014; van Breemen, Dong, & Pajkivic, 2012). In the lower mass region, fragment ions resulting from the cleavage of 9,10- and 15-15'-C-C double bonds were detected (i.e., 177 and 269 *m/z*, respectively). As for lutein, confirmation was made by comparison with an injection of analytical standard of β-carotene. These two identified carotenoids were considered for all further determinations and bioaccessibility experiments (see the following sections).

3.2. Amount of carotenoids in stinging nettles and enriched pasta

The literature shows that levels of carotenoid, like other phytochemicals, in fruit, vegetables and plants are influenced by cultivar, and environmental and agronomic factors, including the maturity stage (Lee, Crosby, Pike, Yoo, & Leskovar, 2005; Lu et al., 2009; Mazza & Cottrell, 2008). Table 1 reports the amount of lutein and β-carotene at four different plant growth stages. Fresh

leaves were sampled at a fixed time of plant development and the carotenoids were extracted and then quantified. The results reveal that the carotenoid content was highest during the flowering phase for both compounds. Lutein and β-carotene were then separately determined in primary ingredients used for pasta (semolina from durum wheat, egg yolk and dried nettle leaves). Dried nettles contained roughly double the amount of carotenoids than the levels found in egg yolk: 52 $\mu g/g$ vs. 23 $\mu g/g$ for lutein; 3.5 $\mu g/g$ vs. 2.2 $\mu g/g$ for β-carotene (see Table 2).

Four different mixtures of semolina, egg yolks and dried nettle together with water (30% w/w) were considered, as described in Table 2, to evaluate the yield of enrichment for the two carotenoids in produced pasta. Effect of dried nettle and egg ingredients was assesses both separately and together. First, the addition of 3.5% w/w of dried nettle to semolina produced similar results to those obtained by mixing semolina and egg yolk (20% w/w) in terms of carotenoid concentration in food matrix. Second, the highest content of lutein and β-carotene was found in egg pasta enriched with nettle. This additive effect allows us to find an almost doubled carotenoid content than non-enriched egg pasta: +44% for lutein (12.68 $\mu g/g$) and +60% for β-carotene (1.07 $\mu g/g$).

Characterization of pasta food matrix was also done with respect to the cooking process. Two types of pasta (P1 and P2) were prepared with (P1-O and P2-O) or without (P1-T and P2-T) nettle enrichment (see section 2.4, Samples, for details). The fraction of carotenoids lost during the cooking process was determined. The largest amount that has been lost occurred in both types of pasta P1-O and P2-O (see table 3), which was around 21% for lutein and 19% for β-carotene. The former carotenoid is more sensitive to cooking process than the latter and the remaining quantities in cooked enriched pasta were still 11% higher for lutein (6.59$\mu g/g$) and 55% higher for β-carotene (1.29$\mu g/g$) with respect to non-enriched egg pasta (see Table 3). This finding is supported by similar behavior described in literature (van Boekel et al., 2010), where the heat treatment (i.e., boiling) is supposed to destroy the molecular complexes between carotenoids and proteins and this denaturation might lead to better carotenoid extraction from the food matrix. For the sake of completeness, experimental standard deviations were not reported in Table 3 to improve reading effectiveness and because RSD% values that

were always lower than 5%.

3.3. Carotenoid bioaccessibility

The bioaccessibility results for lutein and β-carotene from P1 and P2 pasta are reported in Figures 2 and 3, respectively. These values are displayed together with bioaccessibility from nettle capsule supplement. This last food matrix was considered as a reference to compare data obtained from nettle enriched pasta with a suitable food supplement designed to have an easy release of nettle phytochemicals during the digestion process. The bioaccessibility profile for P2-T is not reported because it does not significantly differ from P1-T. The two phytocompounds are characterized by a larger bioaccessibility for lutein than for β-carotene. These results are in accordance with the recent literature (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2009), which emphasized the relationship between carotenoid bioaccessibility and the behavior of chromoplasts (i.e., specialized plant plastids where carotenoids are deposited after their biosynthesize) during the digestion process. Chromoplasts, together with cell membranes, represent a barrier that can affect the release of specific carotenoids from plant tissues (Palmero et al., 2013).

Our findings also revealed that the bioaccessibility for nettle enriched pasta (P1-O and P2-O) is higher after 2 hours of colon fermentation, while for the capsule supplement a longer digestion time (24 hours) was required to reach a maximum level. This clearly suggests a food related factor that influences carotenoid bioaccessibility. Indeed, the effect of the food matrix on phytocompounds bioaccessibility is known from the literature and it is increasingly studied in recent years, including their bioavailability (D'Archivio, Filesi, Varí, Scazzocchio, & Masella, 2010; Johnson, 2013). Additionally, the bioaccessibility trend followed by P1-O and P2-O pasta was similar, even if it was markedly lower for P2-O. Although food additives were supposed to be homogeneously distributed across the pasta sectional area, our results reveal that curly, short, thick shape (i.e., P1) give larger bioaccessibility than straight, long, thin shape (i.e., P2) due to the more efficient digestion. This can be related to the mouth chewing process and, consequently, to the time required for food matrix degradation according to residual food size after the gastric stage. The most interesting feature was found by comparing traditional egg

pasta and nettle enriched pasta. P1-O and P1-T showed very close maximum bioaccessibility for both lutein (about 35% and 40%, respectively) and β-carotene (about 10%) at 2 h colonic fermentation (see Figures 2 and 3). However, their behavior at 24 h and 48 h colonic fermentation was different. Both lutein and β-carotene displayed a still high bioaccessibility at 24 h for P1-T, while for P1-O it decreased from 35% to 20% for lutein and from 10% to 4.5% for β-carotene. In addition, although bioaccessibility did not vary significantly between 24 and 48 h for both P1-T and P1-O in the case of lutein, it converges close to 4% at 48 h for P1-T and P1-O in the case of β-carotene. This can be translated into a more rapid bioaccessibility of lutein and β-carotene from nettle enriched egg pasta than that for non-enriched pasta at an early stage of colonic fermentation (2 h). Non-enriched egg pasta showed a slower bioaccessibility variation, which allowed us to maintain high values at 24 h colonic fermentation for lutein and β-carotene and at 48 h only for lutein (see Figure 2).

All of these aspects require further investigation, particularly in the light of recent advances in understanding the bioaccessibility of carotenoids (Rachel E. Kopec, 2017). Natural structural barriers where carotenoids are stored in vegetable cells seem to play an important role in determining the final bioaccessibility of such phytocompounds (Carrillo, Buvé, Panozzo, Grauwet, & Hendrickx, 2017). In addition, the presence of lipids during the digestion of plant-based foods containing carotenoids, and also micelle formation and nano-, micro- structure of emulsion strongly influence bioaccessibility (Salvia-Trujillo et al., 2017). Other food components, such as oils, or the concentration of intestinal enzymes might affect the overall emulsifying power of the food matrix (Corte-Real et al., 2018; Sotomayor-Gerding et al., 2016). Technological improvement of enriched foods can also derive from encapsulation of antioxidant components to enhance their bioaccessibility (Tan et al., 2014). This work remarks the importance of information that can be found in literature and the effectiveness of continuing with deeper and new insights into functional foods and their bioaccessibility.

4. Conclusions

The outcomes of the present study suggest that Stinging nettle can be a good source of carotenoids, particularly lutein and β-carotene. Dried nettle leaves that are harvested during

flowering growth stage provide large amounts of lutein and β-carotene at 184 and 6.7 $\mu g/g$, respectively, compared to other common food ingredients. This work describes the use of nettle as a functional ingredient for egg pasta. Nettle enriched egg pasta provides for 44% more lutein and 60% more β-carotene than non-enriched pasta. The cooking process produces a loss of between 19% and 21% of the two phytocompounds due to temperature degradation and water boiling. However, nettle enriched egg pasta can provide for 11% more lutein and 55% more β-carotene.

The nutraceutical efficacy of nettle enriched pasta is investigated by means of bioaccessibility experiments for lutein and β-carotene. The gastro-intestinal model reveals that nettle enriched egg pasta behaves differently in terms of carotenoid release from food matrix than nettle food supplement (capsules). The latter shows bioaccessibility profiles with a maximum value after 24 hours of colonic fermentation (47% for lutein and 10% for β-carotene). Conversely, nettle enriched egg pasta displays a maximum bioaccessibility level at lower colonic fermentation time (2 hours). This aspect has additional, unique features that appear when traditional and enriched pastas are compared. Both types have the highest bioaccessibility at an early stage of colonic fermentation (2 hours), but for nettle enriched pasta the bioaccessibility rapidly decays from 35% to 20% for lutein and from 10% to 4% for β-carotene after 24 hours. In contrast, the release of carotenoids from traditional egg pasta is prolonged beyond 24 hours. This suggests that these anti-oxidant compounds are more readily available for intestinal absorption during the early stage of colon fermentation when they are enriched from nettle leaves. High temperature (i.e., water boiling) seems to increase bioaccessibility of lutein and β-carotene in enriched pasta at early stages of digestion, most probably due to degradation of protein-carotenoid complexes.

5. Acknowledgements

This work was partially funded with a contribution (2016) from the University of Ferrara and the Chamber of Commerce, Industry, Artisanship and Agriculture (CCIAA) in Ferrara.
The authors express their gratitude towards Vassalli Bakery S.r.l. for sample production and for fruitful assistance during the experimental work.

The authors thank the Laboratory Terra&Acqua Tech, Member of Energy and Environment Cluster, Technopole of Ferrara (Emilia-Romagna High Technology Network, POR-FESR 2014-2020).

Conflict of interest statement

All authors confirm that there are no conflicts of interest to declare.

Appendix A. Supplementary Material

APCI-MS/MS spectra for the identification of lutein (Figure S1) and β-carotene (Figure S2).

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Figure captions

Figure 1. HPLC-UV/Vis chromatogram of nettle extract recorded at 450 nm.

Figure 2. Bioaccessibility data for Lutein. P1-T: non-enriched type 1 egg pasta. P1-O: nettle enriched type 1 egg pasta. P2-O: nettle enriched type 2 egg pasta. CAP: commercial nettle dietary supplement. Type 1 pasta: curly, short, thick shape. Type 2 pasta: straight, long, thin shape. Letters above bars refer to statistical analysis (see section 2.9) where bioaccessibility values from different sample types were compared for each digestion phase. Values marked with equal letters are not significantly different from each other (P>0.05).

Figure 3. Bioaccessibility data for β-Carotene. P1-T: non-enriched type 1 egg pasta. P1-O: nettle enriched type 1 egg pasta. P2-O: nettle enriched type 2 egg pasta. CAP: commercial nettle dietary supplement. Type 1 pasta: curly, short, thick shape. Type 2 pasta: straight, long, thin shape. Letters above bars refer to statistical analysis (see section 2.9) where bioaccessibility values from different sample types were compared for each digestion phase. Values marked with equal letters are not significantly different from each other (P>0.05).

Figures and Tables

Carotenoid	UD-V	UD-F	UD-S	UD-Q
Lutein	159 ± 9.1^{a}	184 ± 29.5^{a}	51.3 ± 7.7^{b}	77.6 ± 5.9^{b}
β-Carotene	2.3 ± 0.5^{a}	6.7 ± 0.3^{b}	1.8 ± 0.2^{a}	2.8 ± 0.1^{a}

Table 1: Effect of the nettle plant's growth stage on carotenoid content. Concentrations are reported as $\mu g/g$ of dry matter (dm). Column names refer to *U. dioica* plant growth stages: UD-V, vegetative; UD-F, flowering; UD-S, seeds maturity; UD-Q, quiescence. Values for each compound (in row) marked with equal letters in apex are not significantly different from each other (P>0.05).

ingredients	Lutein	β-Carotene
semolina (durum wheat)	6.88 ± 0.21	0.33 ± 0.01
egg	23.12 ± 0.68	2.23 ± 0.06
dried nettle	51.93 ± 1.05	3.51 ± 0.29
mixtures*		
semolina (durum wheat)	4.98 ± 0.08[a]	0.26 ± 0.01[a]
semolina (durum wheat) egg (20% w/w)	8.81 ± 0.20[b]	0.67 ± 0.03[b]
semolina (durum wheat) dried nettle (3.5% w/w)	7.46 ± 0.17[c]	0.42 ± 0.03[c]
semolina (durum wheat) egg (20% w/w) dried nettle (3.5% w/w)	12.68 ± 0.31[d]	1.07 ± 0.04[d]

Table 2: Carotenoid content ($\mu g/g$) in ingredients used for pasta and different kneading mixture. * 30% w/w water. Values for each compound (in column) marked with equal letters in apex are not significantly different from each other (P>0.05).

Sample	Lutein			β-Carotene		
	raw	cooked	loss%	raw	cooked	loss%
P1-T	6.55	5.91	9.77	0.76	0.83	n.a.
P1-O	8.26	6.59	20.21	1.59	1.29	18.87
P2-T	10.80	9.08	15.93	1.01	1.05	n.a.
P2-O	10.66	8.21	22.98	1.51	1.23	18.54

Table 3: Effect of cooking process on Lutein and β-Carotene content in pasta samples ($\mu g/g$ of dry matter). RSD% was alsways smaller than 5% for all determinations.

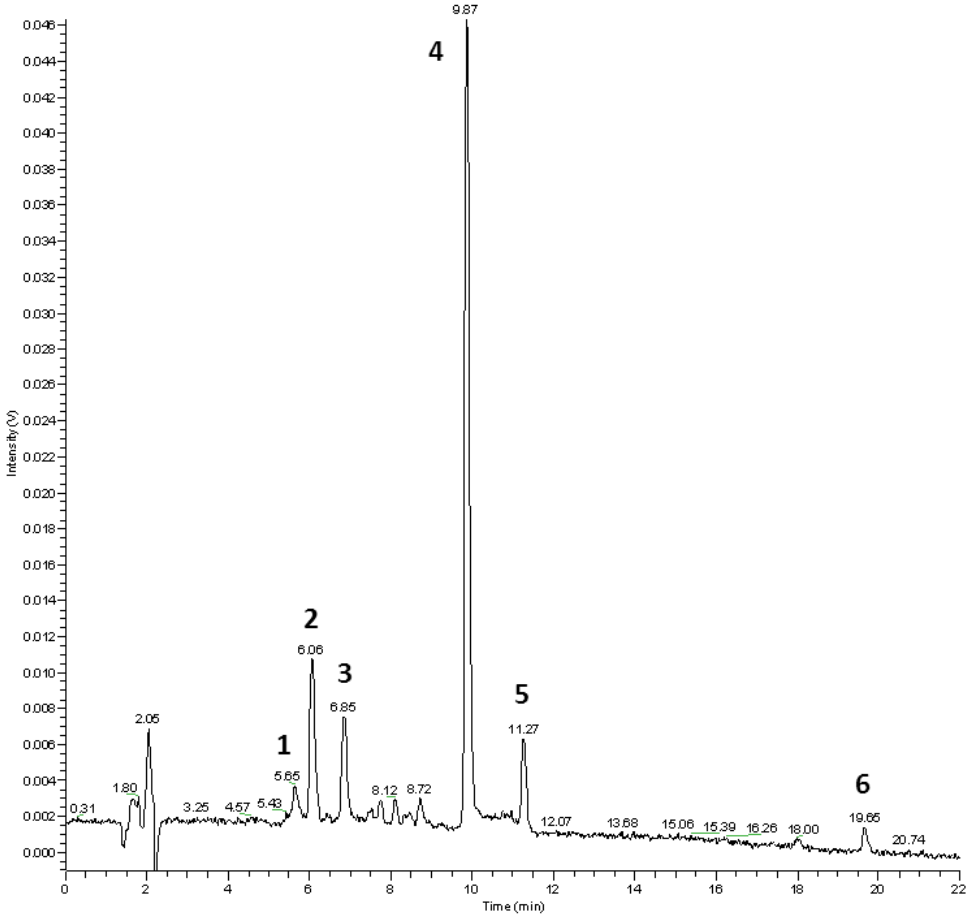
Intensity (V)
Time (min)
1
2
3
4
5
6
0.31
1.80
2.05
3.25
4.57
5.43
5.65
6.06
6.85
8.12
8.72
9.87
11.27
12.07
13.68
15.06
15.39
16.26
18.00
19.65
20.74

Figure 1:

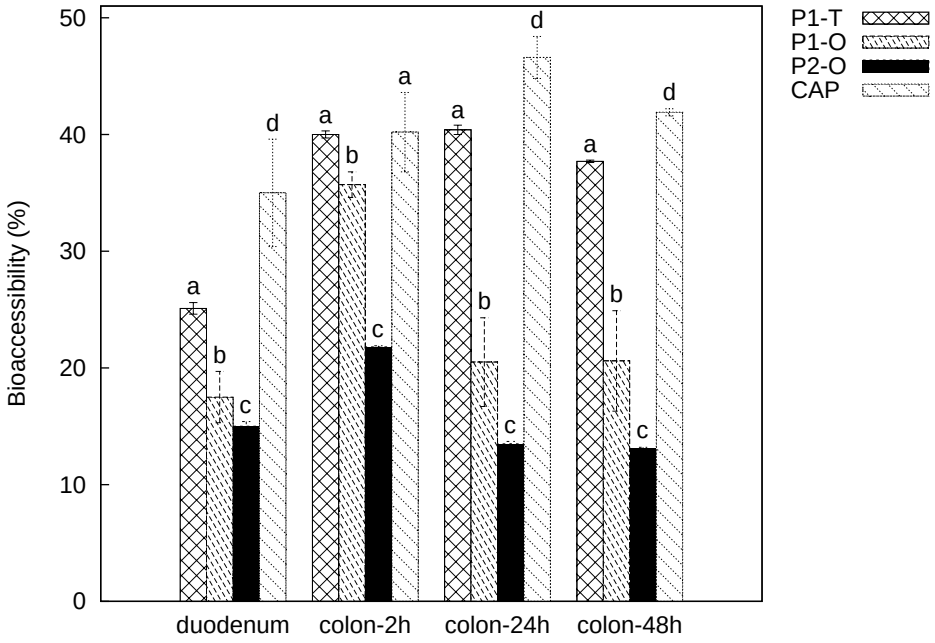
Bioaccessibility (%)
0
10
20
30
40
50
duodenum
colon-2h
colon-24h
colon-48h
P1-T
P1-O
P2-O
CAP
a
b
c
d

Figure 2:

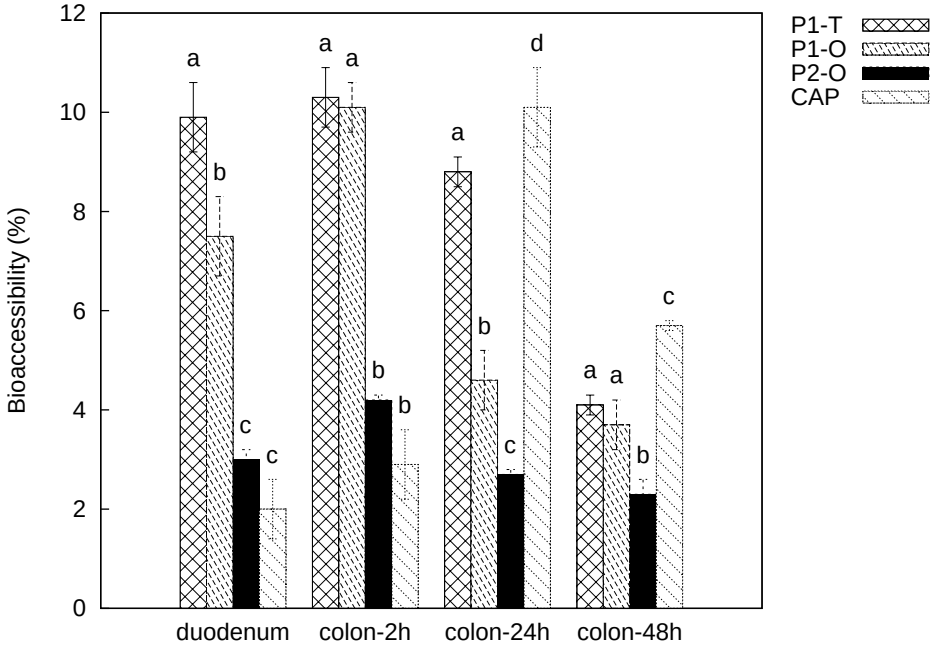
Bioaccessibility (%)
0
2
4
6
8
10
12
duodenum
colon-2h
colon-24h
colon-48h
a
b
c
c
a
a
b
b
a
b
c
d
a
a
b
c
P1-T
P1-O
P2-O
CAP

Figure 3:

Figure 1

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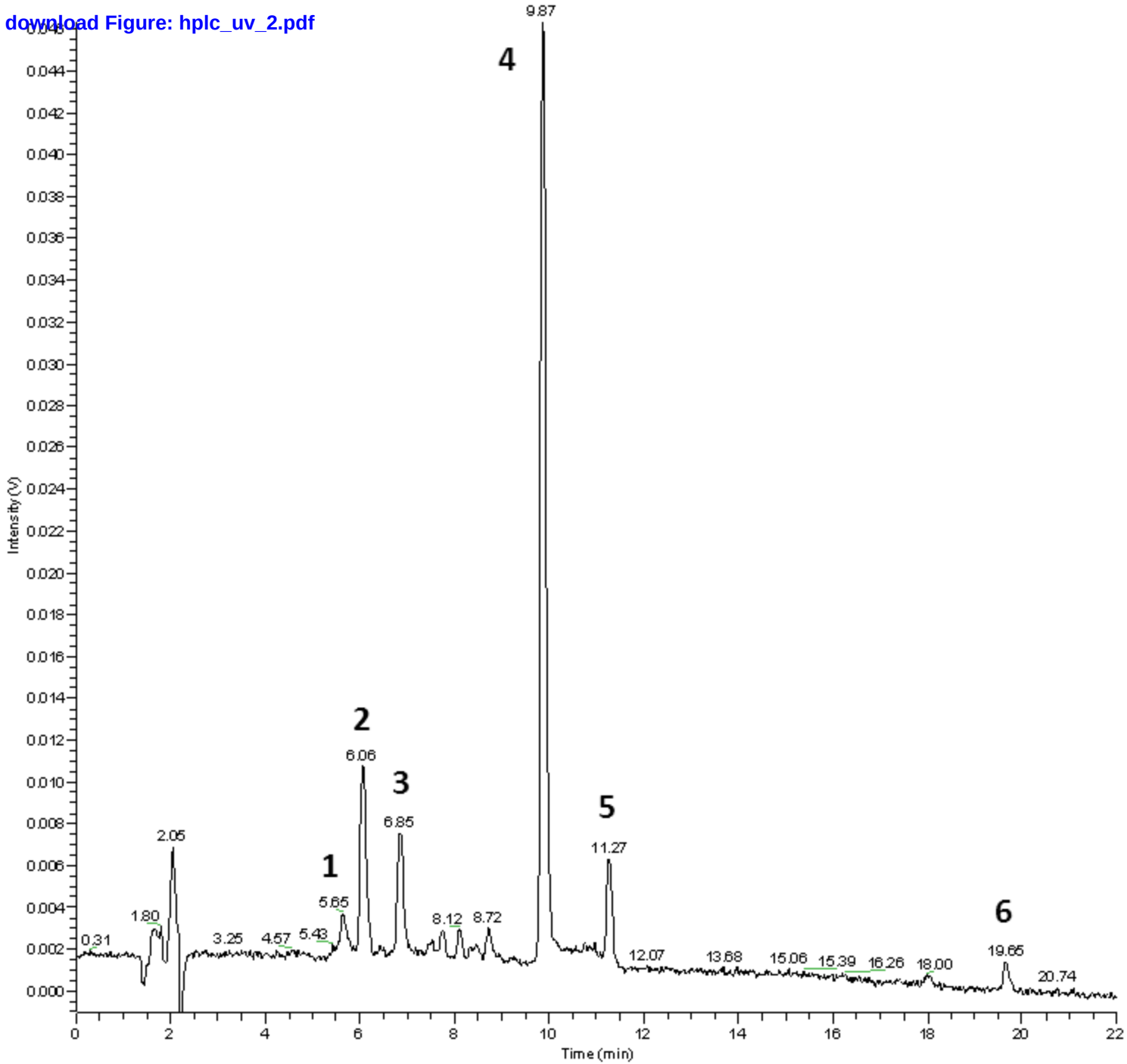

Figure 2

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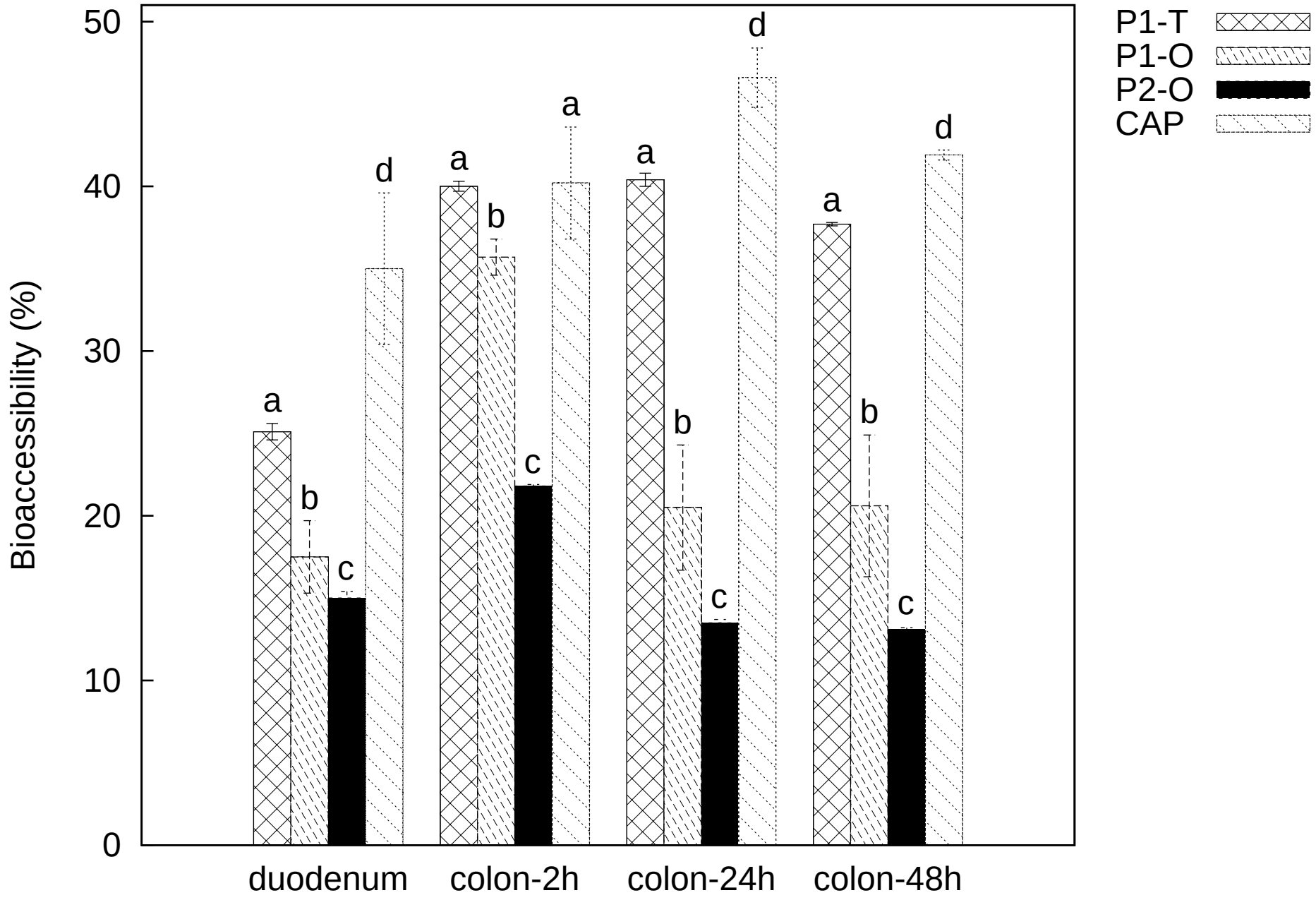

Figure 3

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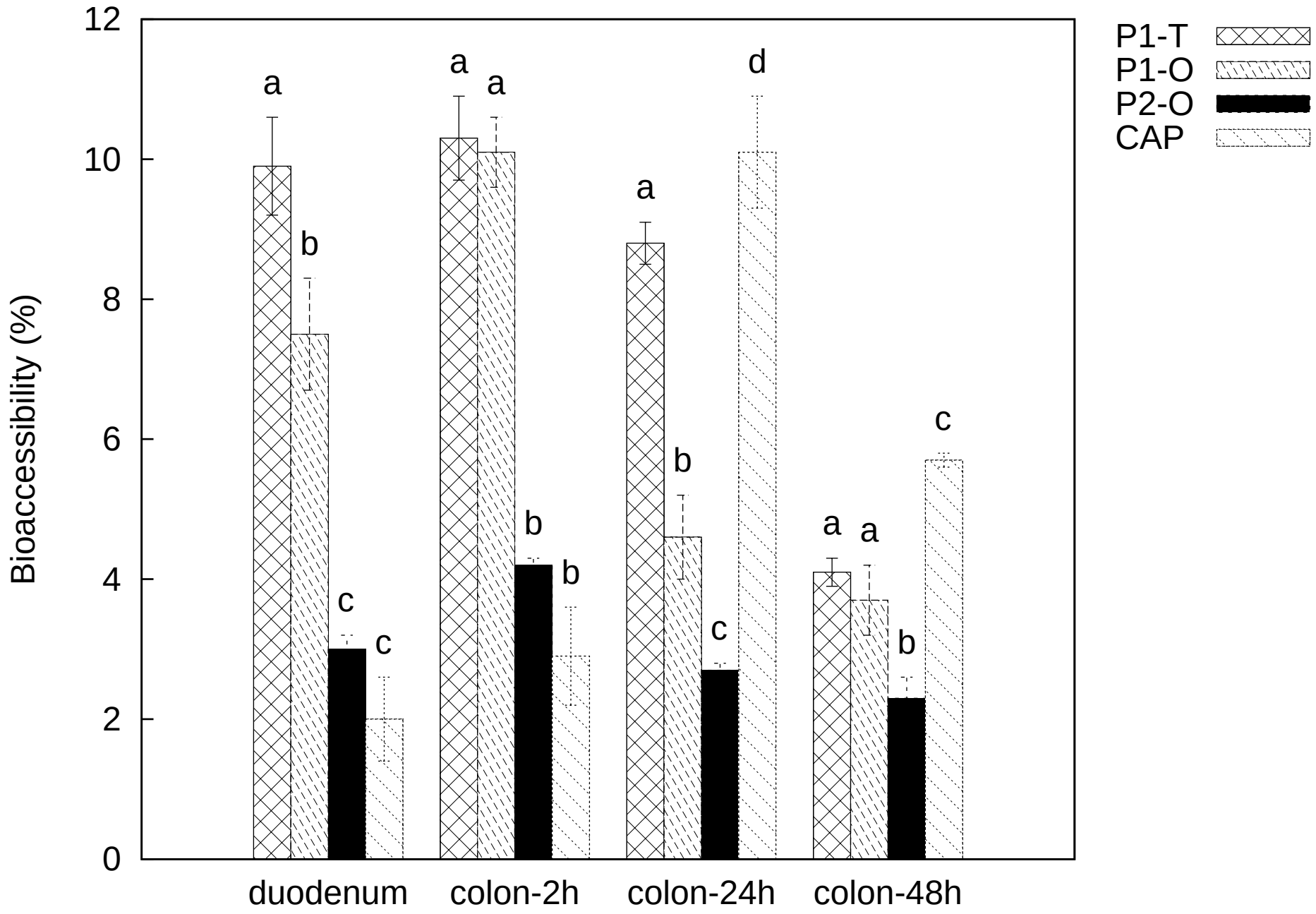

Stinging nettle (*Urtica dioica L.*) as a functional food additive in egg pasta: enrichment and bioaccessibility of Lutein and β-Carotene

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Appendix A. Supplementary Material

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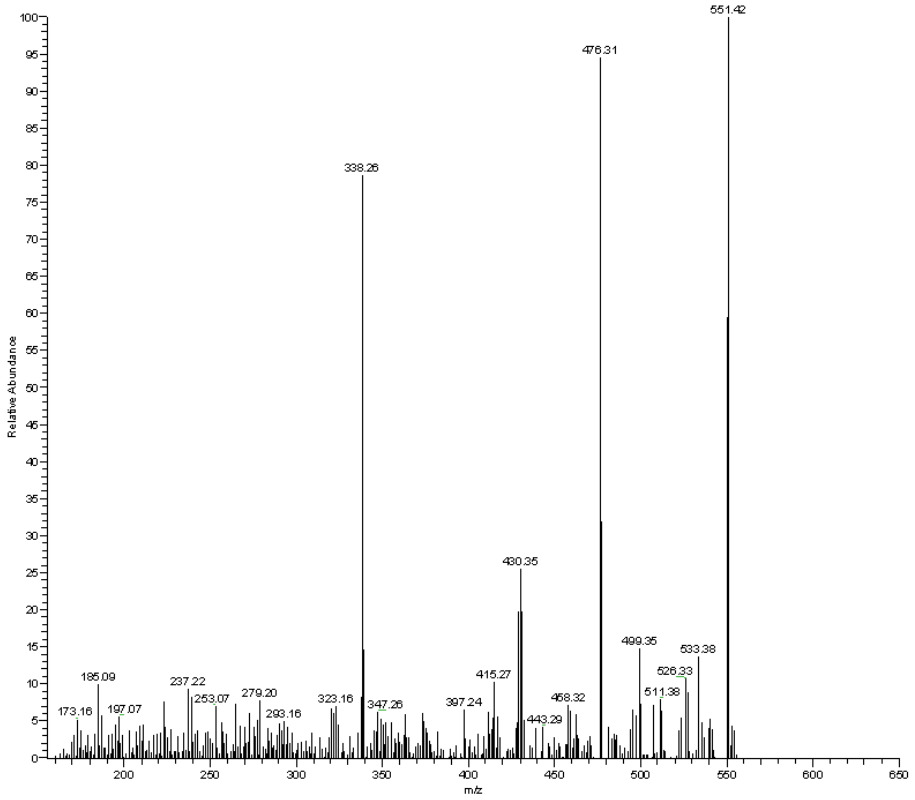


Figure S1. MS/MS spectrum of Lutein from HPLC-UV/Vis-APCI-MS/MS analysis (peak #4, see Fig. 1, main text).

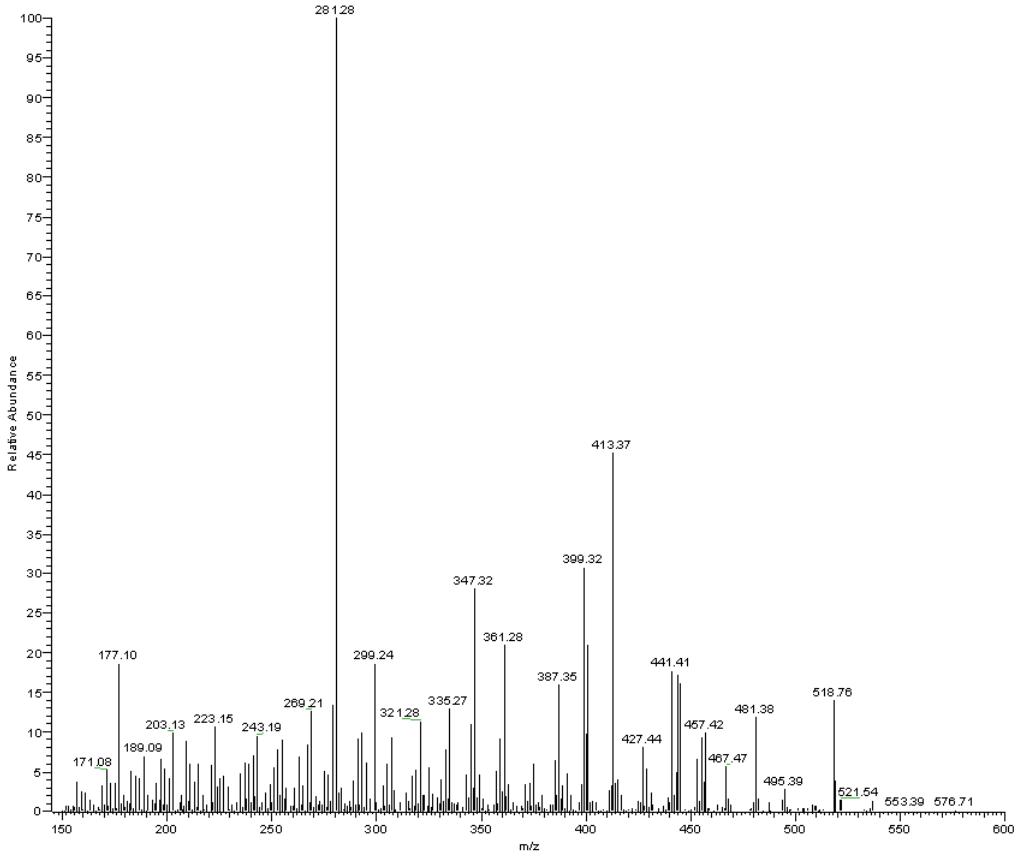
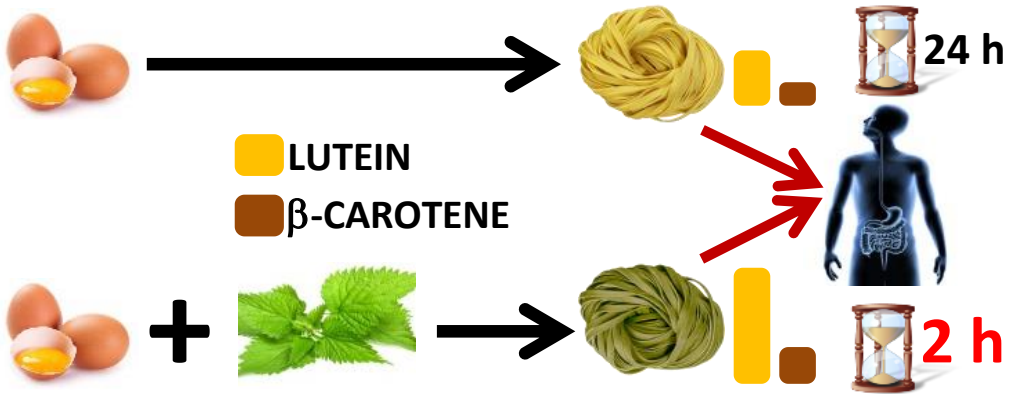


Figure S2. MS/MS spectrum of β-Carotene from HPLC-UV/Vis-APCI-MS/MS analysis (peak #6, see Fig. 1, main text).

*Graphical Abstract



Conflict of interest Statement

All authors confirm that they have read the journal policy on Conflict of interest and that there are no conflicts to declare.

Date: May 9^{th}, 2018

Corresponding author's signature:

Ethical Statement for Journal of Functional Foods

I testify on behalf of all co-authors that our article submitted to Journal of Functional Foods:

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All authors: N. Marchetti, G. Bonetti, V. Brandolini, A. Cavazzini, A. Maietti, G. Meca and J. Manes.

- this material has not been published in whole or in part elsewhere;
- the manuscript is not currently being considered for publication in another journal;
- all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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