Opposite effects of the spinach food matrix on lutein bioaccessibility and intestinal uptake lead to unchanged bioavailability compared to pure lutein

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Abbreviations: SR-BI (Scavenger Receptor class B type I), NPC1L1 (NPC1 transporter like 1), bovine serum albumine (BSA), foetal bovine serum (FBS)

Key words: carotenoid, xanthophyll, lutein isomer, bioaccessibility, *in vitro* digestion, intestine, enterocyte, minipigs.

Abstract

Scope: Food matrix is generally believed to alter carotenoid bioavailability, but its effect on xanthophylls is usually limited. We thus aimed to decipher the digestion-absorption process of lutein in the presence or not of a food matrix. **Methods**: We assessed lutein transfer to gastric-like lipid droplets or artificial mixed micelles when lutein was added to test meals either as a pure molecule ((all-*E*)-lutein) or in canned spinach ((*Z*) + (all-*E*)-lutein). The obtained mixed micelles were delivered to Caco-2 cells to evaluate lutein uptake. Finally, we compared postprandial plasma lutein in minipigs after the two test meals. **Results:** Lutein transfer to gastric-like lipid droplets and to mixed micelles was higher when lutein was added in spinach than when it was added as pure lutein (+614% and +147%, respectively, $p<0.05$). Conversely, lutein uptake was less effective when micellar lutein was from a meal containing spinach than from a meal containing its pure form (-55%, $p<0.05$). In minipigs, postprandial lutein response was delayed with spinach but not significantly different after the two test meals. **Conclusion**: Opposite effects at the micellarization and intestinal cell uptake steps explain the lack of effect of spinach matrix on lutein bioavailability.

Introduction

Lutein is an oxygenated carotenoid belonging to the xanthophyll family. It is mainly provided by green leafy vegetables and egg yolks in the human diet [1, 2]. Lutein selectively accumulates in the retina [3, 4], where it likely protect photoreceptors from blue light [5-7]. Its concentration in this tissue has been negatively associated with the incidence of age-related macular degeneration [8, 9]. These data were recently confirmed in randomized, placebo-controlled clinical trials that have demonstrated that xanthophyll supplementation increases macular pigment levels, improves visual function and decreases the risk of progression to late age-related macular degeneration [10].

Xanthophyll health effects depend on their bioavailability. Their digestion process begins with their dissolution in the fat phase of the meal. This phase is then emulsified into lipid droplets at both gastric and duodenal levels [11, 12]. In the duodenum, xanthophylls are incorporated with lipid digestion products into the mixed micelles [13]. This step, assumed to be indispensable for carotenoid absorption by the enterocytes, can be affected by numerous factors related to the host and to the meal [14], including the food matrix [15]. Mixed micelles then cross the unstirred water layer of the glycocalyx area and theoretically dissociate by pH effect at the brush border level, where micelle components can be picked up by enterocytes. As shown for some other carotenoids [16], lutein uptake by the intestinal cell involves the scavenger receptor Class B type I (SR-BI) [17] and the NPC1 transporter like 1 (NPC1L1) [18].

Degrading and/ or eliminating the food matrix is generally believed to improve carotenoid bioavailability [19]. However, if the deleterious effect of the food matrix has been well described for carotenes, results are less clear regarding xanthophylls [15, 20]. We thus designed a study combining both *in vitro* and *in vivo* models to compare the fate of lutein delivered either as a pure molecule or in canned spinach during the following steps: i) transfer

to gastric-like emulsified lipid droplets, ii) transfer to mixed micelles, iii) uptake by intestinal cells, iv) secretion to the blood stream in minipigs. Canned spinach matrix was specifically chosen because spinach is a good lutein source in human diet [21, 22].

Lutein is usually provided as (all-*E*)-lutein in supplements, whereas it is found as both (all-*E*)- and (*Z*)-lutein in processed or cooked fruits and vegetables - (*Z*)-isomers being produced under thermal processing [23]. We thus assessed (all-*E*)-lutein and both (all-*E*)- and (*Z*)-lutein to evaluate pure lutein and lutein from canned spinach absorption, respectively.

Material and methods

Chemicals

(all-*E*)-lutein (96%) used for emulsion transfer experiments and *in vitro* digestions was purchased from CaroteNature (Mussingen, Switzerland). (all-*E*)-lutein (70% pure) for piglet experiments was from Naturex (Avignon, France). Sunflower oil (Lesieur, France) and canned spinach (Casino, France) were food grade. Sodium acetate and formic acid (HCOOH) were purchased from Merck (Lyon, France). Apo-8'-carotenal (96% pure, used as an internal standard), triolein (65% pure), L-α-phosphatidylcholine and bovine serum albumin (BSA), porcine bile extract and pancreatin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). DMEM containing 4.5 g/L glucose, trypsin-EDTA (500 mg/L and 200 mg/L, respectively), non-essential amino acids, penicillin/streptomycin and PBS were from Life Technologies (Villebon sur Yvette, France). Foetal bovine serum (FBS) was from Dutscher (Brumath, France). Deionized H_2O was purified by a Milli-Q Water Purification system (Millipore, MA, USA). All solvents used were HPLC grade from either SDS (Peypin, France) or Merck.

Lutein transfer to gastric-like mixtures

Preparation and characterization of the different mixtures - Four oil-in-water mixtures, unstabilized and stabilized either by BSA, phospholipids, or a combination of both were prepared to mimic those that exist in the human stomach during digestion of complex meals [24]. All oil-in-water mixtures were prepared by mixing 4 g of sunflower oil in 36 mL sodium acetate buffer 10 mM at pH 4 [12]. For stabilized emulsions either 100 mg of L-α-phosphatidylcholine (PL), 160 mg de serum albumin bovine (BSA), or both were added to the sodium acetate buffer before homogenization. The oil-in-water mixtures were pre-homogenized using a rotor stator homogenizer Silentcrusher at 24000 rpm for 2 min (room

temperature). The obtained crude oil-in-water mixtures were then sonicated using Qsonica sonicator for either 4 periods of 30 s with intervals of 30 s, amplitude 40, 17W, in ice (for BSA- and BSA-phospholipid-stabilized emulsions), or for 4 periods of 60 s with intervals of 30 s, amplitude 80, 25W in ice (for phospholipid-stabilized emulsions). The size distribution of the emulsion lipid droplets was determined in triplicate with a laser particle size analyzer (Mastersizer 2000, Malvern Instruments, Orsay, France). Calculations were performed using the Mie theory with the following measured refractive indexes: 1.475 for oil, 1.333 for the dispersant, and an absorbance value of 0.01 for the emulsion particle. The mean diameter was reported as the surface-weighted diameter d_{32}.

Transfer studies – The transfer efficiency of lutein from either pure lutein or canned spinach to the oil-in-water mixtures was evaluated by the amount of lutein transferred into the oil phase of the mixtures. An aliquot of (all-*E*)- lutein in CH_2Cl_2 (evaporated under argon) or a quantity of spinach puree were placed in a 15 ml glass tube to obtain final amounts of 50 to 100 µg lutein. In order to have a homogeneous matrix, canned spinach was diluted four times with deionized H_2O and pureed using a rotor stator homogenizer Silentcrusher at 16000 rpm for 1 min under dim light at room temperature. Unstabilized or stabilized oil-in-water mixtures (2 mL) were then added to each tube and placed at 37 ° C in the dark for 4 h under vigorous shaking. At defined times, aliquots were harvested, centrifuged for 15 min at 5000 × g, and subjected to one freeze/thaw cycle. Oil was then separated from the aqueous phase of the oil-in-water mixtures by centrifugation for 30 min at 10850 × g. Oil was solubilized in 200 µL of 2-propanol and an adequate volume of CH_2Cl_2/MeOH (v/v) was added to obtain a final volume of 1 mL prior to UPLC analysis. Each experiment was performed in triplicate.

In vitro digestion

The *in vitro* digestion model was run according to [15, 25]. Briefly, 1.2 mg steak, 6.7 mg of boiled potatoes, 200 mg sunflower oil and either 0.196 mg pure lutein or 4 mg of pureed canned spinach (containing the same amount of total lutein) were mixed with 32 mL of NaCl 0.9% (Ultra-Turrax Disperser, 30 sec at 6000 rpm) and 2.5 mL of artificial saliva [26]. The samples were incubated for 10 min in a shaking water bath at 37°C and the pH was then adjusted to 4 ± 0.02 with about 500 µL of 1 M HCl. Then 2 mL of porcine pepsin (40 mg/mL in 0.1M HCl) was added and the mixture was incubated at 37°C in a shaking water bath for 30 min to mimic the gastric phase of digestion. The pH of the partially-digested mixture was raised to 6 ± 0.02 by adding around 800 µL of 0.9M sodium bicarbonate pH 9-10. Then, 9 mL of a mixture of porcine bile extract and pancreatin (containing 3 mg/mL pancreatin and 12 mg/mL bile extract in 100 mM trisodium citrate pH 6.0) and 4 mL of porcine bile extract at 0.1 g/mL were added. Samples were further incubated in a shaking water bath at 37°C for 60 min to mimic the duodenal phase of digestion. The aqueous fraction containing the mixed micelles that were produced upon *in vitro* digestion was separated from oil droplets by centrifugation (2500 × g for 1 h at 10°C), then collected and sequentially passed through a 0.8 µm and a 0.22 µm filter (Millipore, Molsheim, France) in order to obtain a clear solution of mixed micelles. Lutein concentration in the mixed micelles was measured by HPLC and lutein bioaccessibility was expressed either as [lutein recovered into mixed micelles/lutein recovered in the digesta at the end of the digestion × 100] or as [lutein recovered into mixed micelles/lutein initially present into the test meal × 100]. These two calculations were performed to take into account the potential effect of lutein degradation during the in vitro digestions.

Caco-2 cell experiments

Caco-2 clone TC-7 cells were cultured in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated foetal bovine serum, 1% non-essential amino acid, and 1% antibiotics (complete medium) as previously described [27]. For each experiment, cells were seeded and grown during 21 days on transwells to obtain confluent highly differentiated cell monolayers [27]. Twelve hours prior to each experiment, the medium used in apical and basolateral chambers was a serum-free medium. The integrity of the cell monolayers was checked by measuring the transepithelial electrical resistance [17]. At the beginning of each experiment, cell monolayers were washed with 0.5 mL PBS. The apical side of the cell monolayers received the diluted lutein-rich micelles from the *in vitro* digestion experiments (1:5 dilution in DMEM) while the other side received serum-free medium. Cells were incubated for 60 min at 37°C. At the end of each experiment, media were harvested. Cells were washed twice in 0.5 mL ice-cold PBS to eliminate adsorbed lutein, then scraped and collected in 0.5 mL PBS. Preliminary experiment showed that no lutein was found in the basolateral media after 60 min incubation. Absorbed lutein was thus estimated as lutein found in scraped cells. Lutein uptake efficiency was evaluated as the percentage of lutein taken up by cells compared to the initial lutein micellar content or compared to the initial amount of lutein present in the test meal before *in vitro* digestion.

Minipig experiment

All procedures were in accordance with the guidelines formulated by the European Community for the use of experimental animals (2010/63/EU) and the study was approved by the Local Committee for Ethics in Animal Experimentation (CREEA d'Auvergne, Aubière, France). Six adult male minipigs (23.6 ± 0.9 kg body weight) participated in the study. They were housed in individual pens (1 × 1.5 m) separated by Plexiglass walls in a ventilated room with controlled temperature (20–23 °C). Apart from sampling days, they were fed with 400 g

of a commercial feed (Porcyprima, Sanders Nutrition Animale, France). Minipigs were surgically fitted with a permanent catheter (polyvinyl chloride; 1.1-mm i.d., 1.9-mm o.d.) in the aorta allowing repeated blood samplings. Two test meals containing either canned spinach (218 g) or pure lutein in 1.7 g triolein (same amount as that in the 218 g of canned spinach) were given to minipigs according to a cross-over design, with one-week interval. Both contained beef meat (120 g), triolein (30 g), wheat starch (120 g), sucrose (20 g), and cellulose (15 g). Before starting the tests, the animals had been accustomed to receive this type of meals in order to ensure a rapid and complete ingestion of the test meals during the sampling days. On sampling days, blood (5.5 ml) was collected into cold syringes containing heparin (S-Monovettes, Sarstedt, France), before the meal and at 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 10 h after meal ingestion. Samples were immediately centrifuged at 1,500 × g for 10 min at 4°C.

All samples were stored at –80°C under nitrogen before lutein extraction and HPLC analysis.

Lutein extraction and UPLC/HPLC analyses

Emulsion samples and samples for isomer determination – Carotenoids were extracted using the following method from aqueous samples (for emulsion, v = 2 mL; for mixed micelles, v = 0.7 to 0.9 mL; for cells, v= 1 mL; for minipig plasma, v = 0.6 mL). One volume of ethanol containing apo-8'-carotenal as internal standard (final concentration 3.3 µM) was added to the sample with 2 mL hexane. After 10 min stirring at 500 rpm in darkness at room temperature, samples were centrifuged at 2500 g for 10 min at 4°C. The hexanic phase was collected and the aqueous phase was extracted a second time with hexane. The second hexanic phase was pooled to the first one and evaporated under argon. The dryed residue was dissolved in 250 µL of CH_2Cl_2/MeOH (v/v). A volume of 7.5 µL was used for UPLC analysis.

Reversed-phase ultra-performance liquid chromatography (UPLC) were performed on a Waters Acquity™ UPLC system equipped with a diode array detector 190-800 nm (DAD) (Waters Corp., Milford, MA, USA). Empower™ 2 software (Waters) was used for instrument control and integration of chromatograms. Carotenoids were analyzed using an ACQUITY UPLC® HSS C18 column (150 mm × 2.1 mm, i.d. 1.8 µm; Waters Corp., Milford, MA, USA). The column temperature was set at 50°C. The analysis was achieved with a gradient elution using CH_3OH/H_2O (80:20, v/v) (solvent A) and ethyl acetate (solvent B). Gradient conditions of the mobile phase were as follows: A, 100%, 0–2.0 min; A, 100–30%, 2.0–20.0 min; A, 30%, 20.0–22 min; A, 30–20%, 22–23 min, and returned to the initial conditions (solvent A, 100%) for a 5 min re-equilibration period [28]. The system operating pressure was 800 bars at initial gradient conditions. The injection volume was 7.5 µL with partial-loop with needle overfill injection. The retention time and absorption spectral characteristics were used to identify (all-*E*)- and (*Z*)- lutein (Figure 1). The content of lutein was quantified by UPLC DAD analysis with detection wavelength at 470 nm. Quantification was performed by comparing peak areas with standard reference curves.

In vitro digestion, cell and minipig samples - Lutein was extracted from 500 µL aqueous samples using the following method. The carotenoid echinenone was used as internal standard and was added to the samples in 500 µL ethanol. The mixture was extracted twice with two volumes of hexane. The hexane phases obtained after centrifugation (500 g, 5 min, room temperature) were evaporated to dryness under nitrogen, and the dried extract was dissolved in 200 µL methanol/dichloromethane (65/35, v/v). A volume of 10 to 60 µL was used for HPLC analysis. The HPLC system comprised a Thermo-Fisher separation module (LPG-3400SD HPLC Pumps and WPS-3000 TSL Autosampler, Shimadzu, Villebon-sur-Yvette, France) and a DAD-3000 Thermo-Fisher photodiode array detector (detection at 450 nm). Lutein and echinenone were separated using a 250 × 4.6 mm i.d. YMC C30 column

coupled with a 10 × 4.0 mm i.d. C18 5-µm Zorbax guard column (Interchim, Montluçon, France) and kept at constant temperature (35°C) as previously described [29]. Carotenoids were identified by spectral analysis and/or retention time and co-injection compared with pure standards. Quantification was performed using Chromeleon software (version 6.50 SP4 Build 1000, Dionex) comparing peak areas with standard reference curves.

Statistical analysis

Results are expressed as means ± SEM (Standard Error of the Mean). Differences between more than two groups of unpaired data underwent the non-parametric Kruskal-Wallis test. The non-parametric Mann-Whitney U test was used as a post hoc test when the Kruskal-Wallis test showed significant differences between groups. Differences between only two groups of unpaired data were tested by the Mann-Whitney U test. Concerning the minipig study, differences between means obtained after the two test-meals were compared by using paired t-tests. Values of $p<0.05$ were considered significant. All statistical analyses were performed using Statview software, version 5.0 (SAS Institute, Cary, NC, U.S.A.).

Results

Carotenoid composition of canned spinach

The carotenoid content of the canned spinach was in agreement with previous data, confirming that canned spinach is a source of both lutein and β-carotene (4.9 and 4.8 mg /100 g, respectively, Table 1). (*Z*)-lutein represented 44% of total lutein content (Table 2).

Effect of the canned spinach matrix on lutein transfer to gastric-like oil-in-water mixtures

In order to evaluate the transfer of lutein from its food matrix to the gastric-like lipid droplets, we first performed transfer experiments with different types of oil-in-water mixtures. A preliminary experiment performed with an unstabilized oil-in-water mixture, representing the state of dietary lipids at the very beginning of the digestion, showed no difference between the transfer efficiency of pure lutein and that of lutein incorporated in canned spinach (approximately 5% transferred during the first 30 minutes, data not shown). The stabilization of the lipid mixture by BSA led to a better incorporation of lutein from canned spinach compared to pure lutein (5.86-fold increase after 4 h incubation, $p<0.05$, Figure 2A). Similar results were obtained with the emulsion stabilized by phospholipids (2.1-fold increase in favor of lutein from canned spinach, $p<0.05$, Figure 2B). The combination of both BSA and phospholipids, which is the most representative of the physiological conditions, finally showed a maximal incorporation of lutein from spinach (up to 50%) into the oil phase of the emulsion compared to pure lutein (7.1-fold increase, $p<0.05$, Figure 2C).

Effect of the canned spinach matrix on lutein transfer to mixed micelles

Lutein transfer to mixed micelles was investigated after *in vitro* digestion mimicking a buccal, a gastric and a duodenal phase. Figure 3A shows that total lutein from canned spinach was significantly better incorporated into mixed micelles than pure lutein (+35% when lutein bioaccessibility was expressed as the ratio of lutein recovered into mixed micelles to lutein recovered in the digesta, $p<0.05$). This effect was more pronounced when lutein bioaccessibility was expressed as the ratio of lutein recovered into mixed micelles to lutein initially present in the meal: + 147% ($p<0.05$, Figure 3B). This was likely due to the fact that lutein was more degraded during the digestion of the meal containing pure lutein than during the digestion of the meal containing spinach (+ 48.1% of total lutein recovered in the digesta from the meal containing spinach compared to the meal containing pure lutein).
The ratio [(*Z*)-lutein/total lutein] was significantly lower in mixed micelles compared to canned spinach (Table 2).

Pure lutein and canned spinach lutein uptake by Caco-2 cells

We used diluted mixed micelle solutions obtained from the *in vitro* digestions to perform this experiment. Figure 3C shows that micellar lutein obtained from the digestion of pure lutein was better absorbed than micellar lutein obtained from the digestion of canned spinach (+55%, $p<0.05$). As shown in Table 2, the ratio [(*Z*)-lutein/total lutein] was not significantly different in cell cytosol compared to mixed micelles.

Overall, if we expressed the uptake efficiency as the ratio of lutein recovered in the cell compartment compared to the lutein initially present in the meals, no significant difference was observed between pure lutein and lutein from canned spinach. Indeed the uptake efficiency of lutein by a well of Caco-2 cells after 1 h incubation was 0.68 ± 0.09 % and 0.56 ± 0.07 % for pure and canned spinach lutein, respectively.

Effect of the canned spinach matrix on lutein bioavailability in minipigs

Postprandial plasma lutein concentrations over time, which were used to estimate lutein bioavailability, showed a peak at 2-3 h after the meal containing pure lutein. This peak was delayed at 6 h after the meal containing spinach (Figure 4A). However, postprandial plasma lutein responses (expressed as area under the postprandial 0-10 h curves) after both meals were not significantly different (101.4 ± 14.5 nmol.h/L and 100.3 ± 13.3 nmol.h/L for pure and canned spinach lutein, respectively; Figure 4B). No significant discrimination was observed between (*Z*)- and (all-*E*)-lutein isomers in minipigs as the ratio [(*Z*)-lutein/total lutein] in blood remained not significantly different from the ratio observed either in the canned spinach or in the mixed micelles or in the Caco-2 cells compartment (Table 2).

Discussion

This study aimed at explaining why lutein biovailability is apparently less impacted by food matrix than the bioavailability of the two-other main dietary carotenoids, i.e. lycopene and beta-carotene [15, 20].

It is true that the food matrix has been described several times as a factor impacting both lutein transfer to mixed micelles and absorption, based on the observation that these parameters can significantly vary from one vegetable to another [30, 31]. However, the effect of food matrix disruption on lutein bioaccessibility led to conflicting results. A first study showed that reduction of particle size resulted in a three- to fourfold increase in liberation of lutein when comparing whole leaf and puree preparations of spinach [32], although a subsequent thermal treatment could abolish this positive effect. Another study highlighted that the xanthophyll β-cryptoxanthin bioaccessibility was higher from juice than from fresh fruits despite a pasteurization step [33]. These results are not consistent with a human study during which subjects received, over a 3-wk period, either a control diet or a control diet supplemented with carotenoids or one spinach product including whole leaf spinach with an almost intact food matrix, minced spinach with the matrix partially disrupted and enzymatically liquefied spinach [20]. Authors showed no effect of the food matrix and its disruption on lutein response, but these two parameters negatively impacted response in β-carotene, which displayed a lower bioavailability compared to lutein. Van het Hof and colleagues also compared the efficacy of spinach and chopped spinach (*i.e.* 300 g/d during 4 d) in increasing plasma levels of lutein [31]. In this work, the disruption of the spinach matrix did not enhance the bioavailability of lutein either.

In our study, we specifically dissociated the micellarization step from the uptake by enterocytes step. Our data highlight that lutein transfer to mixed micelles, i.e. lutein bioaccessibility, was significantly higher in the presence of spinach matrix, and this observation was also made at the initial step of the transfer from the food matrix to the gastric emulsion.

A first hypothesis to explain this result would be that (*Z*)-lutein present in spinach was more bioaccessible than pure (all-*E*)-lutein. However, this is unlikely because as shown in Table 2, [(*Z*)-lutein/total lutein] ratio was significantly lower in mixed micelles than in canned spinach, suggesting a preferential incorporation of (all-*E*)-lutein compared to (*Z*)-lutein. Several spinach components have previously been described as impacting lutein bioaccessibility. Surprisingly, most of them are supposed to negatively affect it. For instance, it is widely acknowledged that carotenoids compete between each other for their transfer to mixed micelles [34]. We could thus expect a competition between spinach lutein and β-carotene. Moreover, as fat-soluble vitamins also compete between each other for micellar incorporation, and as spinach is a major source of vitamin K (462 µg/100 g of drained product [22]), we could also expect a competition between lutein and vitamin K [35]. However, we did not observe such interaction, which is consistent with previous *in vitro* results showing that these competitions may only affect carotene transfer from emulsion lipid droplets to micelles, and not that of xanthophylls [36]. Fibers are also supposed to modulate carotenoid micellarization (drained canned spinach contain about 2.4 g/100 g of dietary fibers [22]). It has been shown that medium- and high-viscosity alginates and pectins could inhibit lutein micellarization *in vitro* [37]. Such effect that was also observed in another *in vitro* study using oat bran, wheat bran, and pectin [38], and a clinical study confirmed that different types of dietary fibers could significantly reduce lutein postprandial response [39]. However, Castenmiller and colleagues showed in humans that the addition of both soluble fibers and

pectin (10 g/kg wet weight) to enzymatically liquefied spinach to test meal consumed over a 3-wk period had no effect on lutein response [20], which suggest that fiber effect may be moderate, and which is reliable with our data. Finally, a last factor that could interact with lutein transfer to mixed micelles may be presence of calcium [40, 41] (about 126 mg/100 g of canned spinach [22]). However, consistently with our results, the negative effect of divalent cations seems to be nonsignificant at normal pancreatin and bile concentrations, and even at high doses, calcium appeared not to have any significant effect on spinach lutein absorption in humans [42].

Conversely, some other factors present in spinach, such as antioxidants, could positively impact lutein bioaccessibility. Indeed, by protecting micellar lutein degradation, they may lead to a higher final micellar lutein content. Canned spinach main antioxidants are other carotenoids, as well as vitamin E (1.25 mg/100 g [22]). As we recovered significantly more lutein at the end of the *in vitro* digestion process with the test meal containing lutein from spinach than with the meal containing pure lutein, we suggest that a significant effect of spinach matrix during the digestion process is a protection of lutein molecule.

We then showed that lutein included in micelles isolated from spinach *in vitro* digestion was significantly less absorbed than lutein included in micelles obtained from pure lutein digestion. Conversely to previous data [43], this result was independent of lutein isomerization, as [(*Z*)-lutein/total lutein] ratio remained similar in Caco-2 cells compared to mixed micelles.

Most of the aforementioned factors can also impact carotenoid absorption by the intestinal cells. For example, competitions for absorption between lutein and other carotenoids or fat-soluble vitamins have been previously described *in vitro* [17] and *in vivo* [44, 45]. A competition with β-carotene is thus very likely in our study. Although competition between

vitamin K and lutein has not been assessed yet, we suggest that it can contribute to the significant reduction of spinach lutein absorption by intestinal cells in culture compared with pure lutein. Indeed, lutein and vitamin K share common absorption pathways via SR-BI [46, 47] and NPC1L1 [18, 48]. Besides, the total polyphenols content of spinach may range between 150 and 200 mg/100 g [49]. Polyphenols such as naringenin have been described to decrease lutein uptake by Caco-2 cells [50]. We thus suggest that spinach polyphenols may also participate in the observed lutein uptake inhibition.

When we combined both the positive effect of the spinach matrix on lutein bioaccessibility together with the negative effect of this matrix on lutein uptake by Caco-2 cells, we found no significant effect of spinach food matrix in lutein overall bioavailability. This result was confirmed *in vivo* in minipigs. However, although there was not significant effect of the food matrix on the amount of lutein absorbed, there was a significant effect of this matrix on the kinetic of lutein absorption as suggested by the lutein absorption peak, which was delayed in the presence of the food matrix. This delay may be due to the fact that lutein from spinach should first be extracted from its matrix to be available for absorption. The putative effect of such delay in terms of lutein bioavailability in target tissues or antioxidant properties of plasma during the postprandial phase need to be further investigated.

Overall, we showed that the absence of effect of the food matrix on lutein absorption is due to an opposite effect of this matrix at the digestion and the absorption steps: food matrix seems to protect lutein from degradation and to favor its transfer to mixed micelles, while it impairs lutein uptake by the enterocyte. This effect was independent from the lutein isomerization observed during canning. This study highlights the importance of coupling both bioaccessibility and absorption studies to evaluate fat-soluble micronutrient bioavailability.

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Figure legends

Figure 1: Chromatogram of canned spinach extract

Analytical conditions are described in the Material and Methods section. Major pigments identified are: (all-*E*)-lutein (1), (*Z*)-lutein (2), β-carotene (3), unidentified carotenoids (4 and 5), chlorophylls (a, b, c, and d), internal standard, i.e. apo-8'-carotenal, (IE).

Figure 2: **Effect of the canned spinach matrix on lutein transfer to gastric like oil-in-water emulsions**

A: Lutein transferred to emulsion stabilized by BSA

B: Lutein transferred to emulsion stabilized by phospholipids

C: Lutein transferred to emulsion stabilized by BSA +phospholipids

Data are means ± SEM of 3 assays. Spinach lutein (●), pure lutein (○).

Figure 3: Effect of the canned spinach matrix on lutein bioaccessibility and lutein uptake by Caco-2 cells

0.196 mg pure lutein or 4 mg of canned spinach (containing 0.196 mg lutein) were added to test meals in *in vitro* digestion experiments.

A: Lutein transferred to mixed micelles at the end of the digestion was calculated as the percent of lutein that was recovered in the micelle rich fraction relative to the amount of lutein remaining in the digesta at the end of the digestion.

B: Lutein transferred to mixed micelles at the end of the digestion was calculated as the percent of lutein that was recovered in the micelle rich fraction relative to the amount of lutein that was initially present in the meal.

C: Mixed micelle rich fractions obtained after *in vitro* digestion of the different sources of lutein were incubated at the apical side of Caco-2 cell monolayers and lutein uptake efficiency (% of lutein recovered in the cells after 1 h incubation relative to the amount of lutein that was initially present in the micelle fraction) was calculated.

Bars represent means ± SEM of 3 measurements. An asterisk indicates a significant ($p<0.05$) difference between means showed in each graph.

Figure 4: Effect of spinach matrix on postprandial plasma lutein responses in minipigs.

A: plasma lutein concentrations over the postprandial period.

Minipigs (n=6) received two test meals containing either spinach (218 g, ●) or pure lutein (same amount than in the 218 g of spinach, ○) according to a cross-over design, with one-week interval. Data are means ± SEM. An asterisk indicates a significant ($p<0.05$) difference between two means obtained at the same time.

B: Areas under the curves showed in figure 4A.

Tables

Table 1: Carotenoid composition of canned spinach.

Carotenoids	Spinach content (mg/100 g of wet product)
Total lutein	4.9 ± 0.3
All-(*E*) lutein	2.8±1.2
(*Z*)-lutein	2.1±0.1
β-carotene	4.8±0.6

Data = means ± SEM (n = 13).

Table 2: Proportion of (all-*E*)- and (*Z*)-lutein at the different steps of the digestion-absorption process of canned spinach.

	(all-*E*)-lutein	(*Z*)-lutein	(*Z*)-lutein/ Total lutein
Canned spinach (mg/100 g of wet product, n=13)	2.8±1.2	2.1±0.1	0.44±0.01[a]
Mixed micelles (ng/mL, n =4)	1498 ± 36	763 ± 35	0.34±0.01[b]
Caco-2 cellular content after 1 h incubation (ng/mL, n = 3)	18 ± 1	9 ± 2	0.33±0.02[b]
Minipig plasma 3.5 h after the test meal (ng/mL, n = 4)	13 ± 5	7 ± 2	0.30±0.21[a,b]

Data = means ± SEM. Different letters indicate a significant difference between the means.

Figure 1

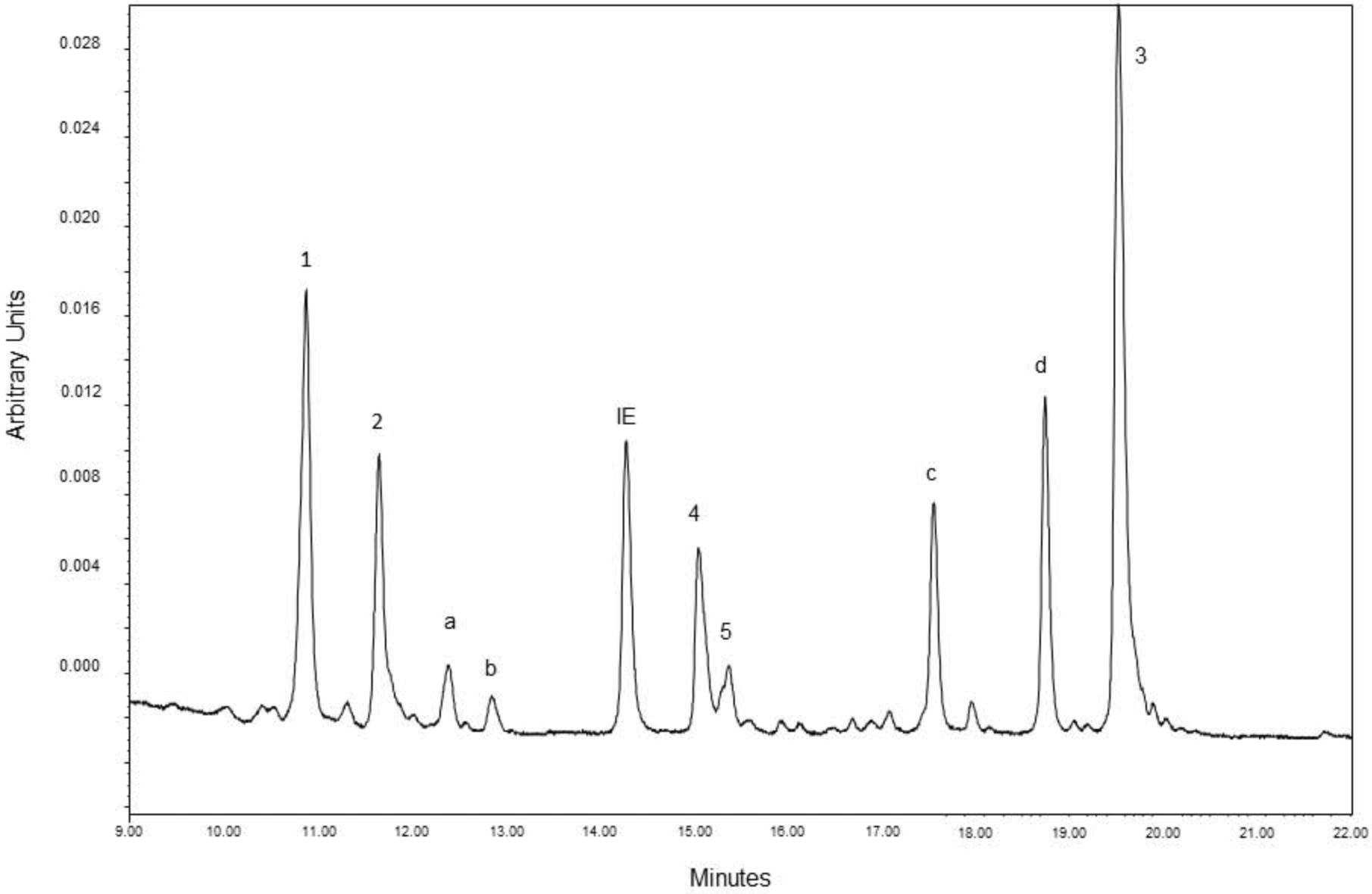

0.028
0.024
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0.012
0.008
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0.000
Arbitrary Units
1
2
a
b
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3
9.00
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22.00
Minutes

Figure 2

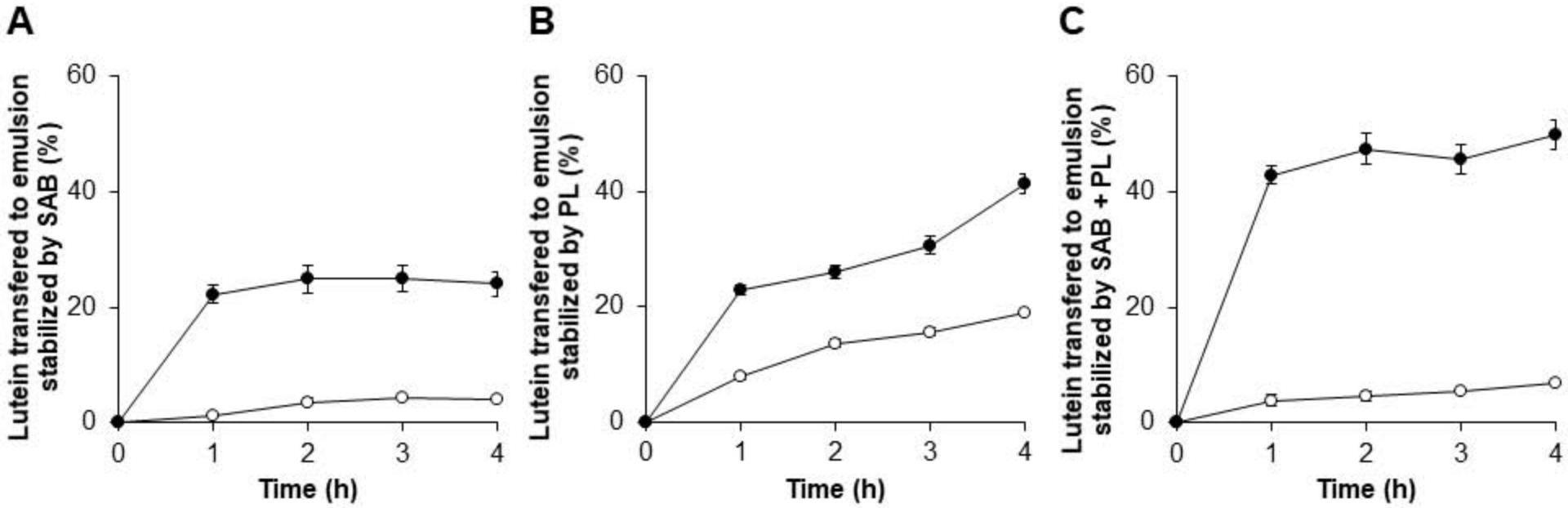

A
B
C
Lutein transfered to emulsion stabilized by SAB (%)
Lutein transfered to emulsion stabilized by PL (%)
Lutein transfered to emulsion stabilized by SAB + PL (%)
0
20
40
60
0
1
2
3
4
Time (h)

Figure 3

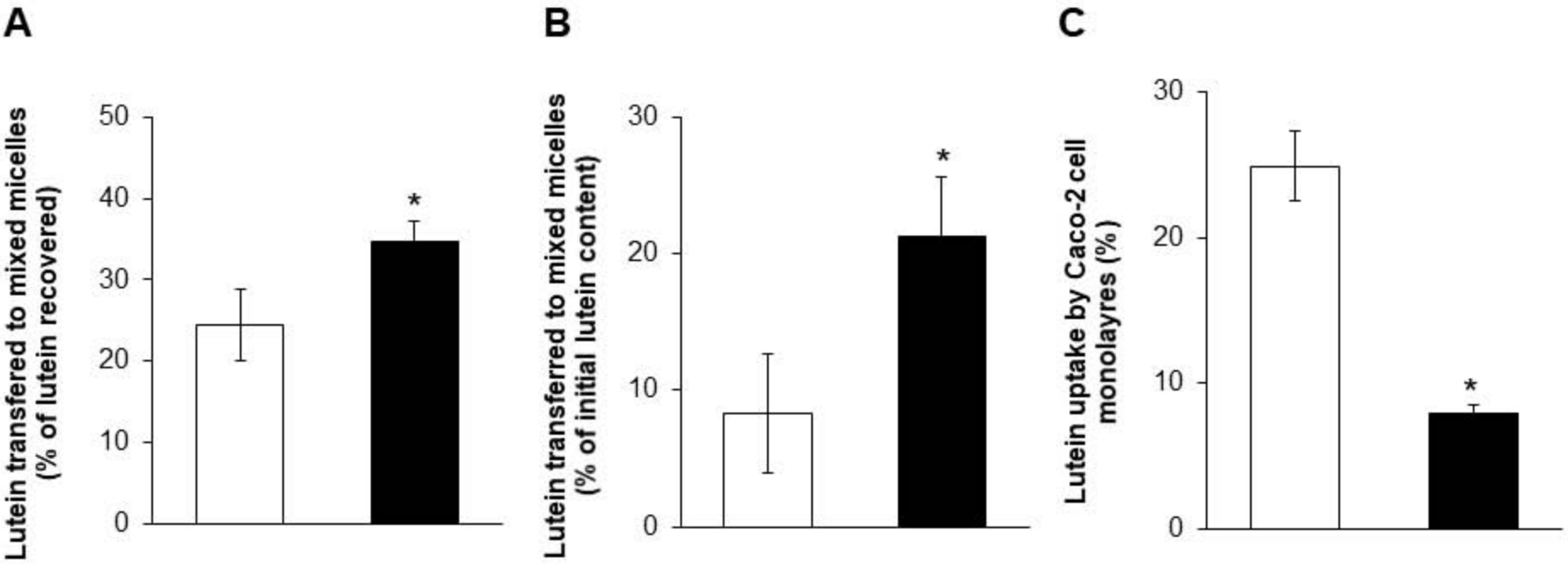
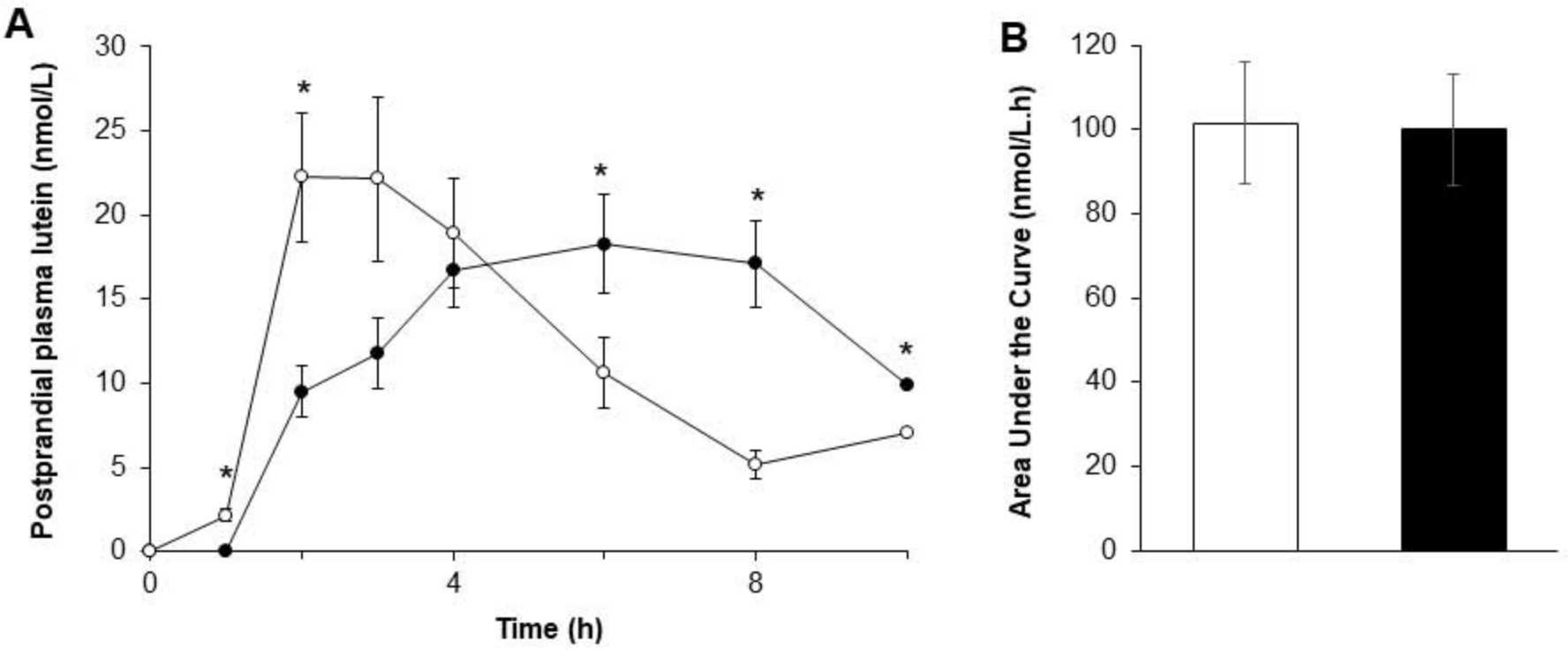

A
Lutein transfered to mixed micelles (% of lutein recovered)
50
40
30
20
10
0
*
B
Lutein transferred to mixed micelles (% of initial lutein content)
30
20
10
0
*
C
Lutein uptake by Caco-2 cell monolayres (%)
30
20
10
0
*

Figure 4


A
Postprandial plasma lutein (nmol/L)
30
25
20
15
10
5
0
0
4
8
Time (h)
*
*
*
*
*
B
Area Under the Curve (nmol/L.h)
120
100
80
60
40
20
0