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*In vitro* bioaccessibility of lutein from cupcakes fortified with a water-soluble lutein esters formulation.

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## Highlights

Lutein esters are stable to baking process during production of fortified cupcakes

Activity of pancreatic lipase towards lutein esters was low after *in vitro* digestion

The efficiency of xanthophyll bioaccessibility reached significant values (30-80%)

## Abstract

Bioaccessibility is a key factor in the successful development of functional foods, particularly when the food matrix is quite different from that of the natural source(s) of the target bioactive ingredient. Although staple foods contain xanthophylls, the amounts are relatively low to achieve the desired beneficial effect in health, and during baking degradative reactions may contribute to reduce such amounts. The addition of water-soluble formulation of lutein as an ingredient in the cupcake recipe to fortified the amounts of this xanthophyll in the final product, showed satisfactory stability degree and resistance to the baking process. Indeed, the *in vitro* bioaccessibility reached adequate efficiency levels, ranging 30-80% of the initial lutein content. Activity of pancreatic lipase over lutein esters was low, but the hydrolysed lutein was completely incorporated into micelles. Indeed, the main micellar lutein

content remained in its esterified form what could facilitate subsequent hydrolysis by other mucosal hydrolytic enzymes. These results point to fortified staple foods as functional foods that may help to diminish the risk of degenerative processes related with deficient lutein intake/incorporation in humans.

Keywords: baked products; bioaccessibility; fortification; functional food; stability of lutein; carotenoids; lutein esters; *in vitro* digestion model; food analysis; food composition.

Chemical compounds studied in this article

Lutein (PubChem CID: 5281243); Acetone (PubChem CID: 180); Calcium chloride (PubChem CID: 5284359); Hydrochloric acid (PubChem CID: 313); Sodium hydroxide (PubChem CID: 14798); Sodium chloride (PubChem CID: 5234); Sodium bicarbonate (PubChem CID: 516892); Diethyl ether (PubChem CID: 3283); Ethanol (PubChem CID: 702).

## 1. Introduction.

Eye-health disorders are among the growing global concerns. The World Health Organization (WHO) has provided estimates of visual impairment and identified several causes of blindness, which include cataract, glaucoma and age-related macular degeneration (AMD) as the leading sources (WHO, 2012). Incidence of AMD and cataract is expected to rise in the next years and public health agencies claim for the development of strategies based on diet and supplements to reinforce the intake of the food compounds known to decrease risk of these eye-health illnesses (Abdel-Aal et al., 2013). Lutein is the primary dietary xanthophyll pigment responsible for macular pigment optical density in primates. A low intake of lutein food sources is related with the risk of developing AMD (Dwyer et al., 2001; Seddon et al., 1994; Shao, 2001). In fact, the retinas of AMD patients have reduced lutein content, and increased lutein intake to enhance the macular pigment optical density has been already evidenced to play a preventive and therapeutic role (Pratt, 1999; Richer et al., 2004; Snodderly, 1995). Thus, an intake of 6 mg/day of macular carotenoids from foods may

decrease the risk of developing AMD (Seddon et al., 1994). Commonly consumed green vegetables like kale, parsley, spinach and collards are excellent sources of lutein and zeaxanthin (Humphries, & Khachik, 2003), that also deposits in the human retina (Bone et al., 1988). Consumption of these and other lutein/zeaxanthin sources accomplishes an estimated daily intake ranging 1-4 mg of xanthophylls (Lucarini et al., 2006; Manzi et al., 2002).

However, before accumulation of these xanthophylls in the retina and development of any beneficial activities, they must be liberated from the food source, absorbed by intestinal epithelial cells and transported to the target tissue. Due to the lipophilic nature of carotenoids, they must be incorporated into mixed micelles, once they are released from the food matrix, where carotenoids are carried to the intestinal epithelium. Several factors extremely influence the effectiveness of both digestion (micellarization) and uptake by enterocytes, including physicochemical properties, food matrix composition and processing level, interaction with other dietary compounds, nutritional status, gut health and genotype of the host (Castenmiller & West, 1998; Yonekura & Nagao, 2007). Regarding the effects of food matrix on micellarization, this is one of the factors that can be assessed applying the *in vitro* approaches developed in the last years, which provide significant insights on the multifactorial setup from which carotenoid bioaccessibility results (Garret et al., 1999; Granado-Lorencio et al., 2007; Rodriguez-Amaya, 2010). Thus, with these methods it has been shown that micellarization of carotenoids is relatively low from fruits and vegetables in comparison with that one reached by supplements, while fruits offer a higher efficiency in the micellarization step than vegetables, which show a high variability in the transfer process from digesta to micelles (O'Connell et al., 2007). Several other studies of *in vitro* bioaccessibility of xanthophylls from foods different than fruit and vegetables report a wide range of efficiency values, in terms of percentage (Garret et al., 2000; Reboul et al., 2006; Werner & Böhm, 2011; Xavier et al., 2014; Gumus et al., 2016). The application of these results comprehends from understanding the digestive processes, including the enzymatic hydrolysis involved in xanthophyll esters availability (Chitchumroonchokchai & Failla, 2006), or for comparative purposes among different food sources, but also for an efficient design of new food delivery systems. Indeed, measurement of bioaccessibility is relevant to determine the suitability of the integration of a target compound (carotenoids) in formulations, functional foods, or food matrices where that compound is not naturally found or to reinforce its content (Fernández-García et al., 2009).

This is the case of staple foods that present low amounts of carotenoids in comparison with fruit and vegetables, at least in those favoured varieties for human consumption; the

white flour is perceived as pure and uncontaminated, while yellow flour is considered for animal feed (Graham & Rosser, 2000). However, there are reasons to consider staple foods in general, and particularly wheat-based products like bread, pasta, cookies, and cupcakes, as suitable delivery systems for carotenoids that can bring health benefits to humans. First, wheat-based products are produced with simple ingredients and during processing could easily include key ingredients (lutein) in the formulation. These products are widely consumed around the world, being a significant contributor to the daily food intake. Although some wheat species present relatively high lutein contents (5.4-7.4  $\mu\text{g/g}$ ) their xanthophyll content decreases significantly during processing, with average carotenoid losses ranging from 21% to 49% depending on manufacturing conditions and the kind of preparation (bread, pasta or water biscuit; Hidalgo et al., 2010). Consequently, it is necessary to add extra lutein amounts to compensate the losses of this pigment during processing or storage.

Development of fortified lutein products is of interest to the food industry as such new products would increase the variability of lutein sources. Nevertheless, it is necessary to provide scientific evidences to assure their effectiveness mainly in terms of bioaccessibility of lutein. The present study aims to determine the *in vitro* bioaccessibility of lutein in cupcakes fortified with different concentrations of a water-soluble lutein esters formulation. A

## 2. Material and methods.

2.1. Raw materials. The cupcake recipe includes white flour, baking powder, margarine, skimmed milk, eggs, and water that were acquired in a local supermarket. Cupcakes were formulated with different amounts of Vegex Lutein WS® water-soluble lutein formulation (0.3% w/v) for food purposes (Christian Hansen, Horsholm, Denmark). Besides lutein, the formulation contains modified starch, sunflower oil, maltodextrin, ascorbic acid, ascorbyl palmitate, alpha-tocopherol, and sodium benzoate.

2.2. Chemicals and solvents. Acetone and water (HPLC grade) were provided by Romyl (Teknokroma, Barcelona, Spain). All other solvents were of analytical grade.  $\alpha$ -Amylase, pepsin, bile extract, pancreatin, and lipase from porcine pancreas were obtained from Sigma (St. Louis, USA).

2.3. Preparation of cupcakes. The preparation of cupcakes was made in a similar fashion to the recipe described by Abdel-Aal et al. (2010) but with slight modifications. The cupcakes formulation according baker's percentage was the following: 100% of wheat flour, 110% of sugar, 3.75% of baking powder, 57.5%

of margarine, 75% of skimmed milk, and 60% of eggs. The necessary amount of water-soluble lutein formulation to reach a final lutein content of 1, 2, 3, 4, 6, 12 mg of lutein per serving was added to the base flour. One serving was considered as 1 unit of cupcake (weight:  $65 \pm 1$  g; diameter: 7 cm; height: 2.8 cm). Baking was performed at 180 °C for 20 min. At least 3 cupcakes per lutein fortification level were baked as well as 3 cupcakes with no lutein addition. Baked products were stored at -20 °C until analysis and application of digestion method.

- 2.4. *In vitro* digestion method. The experimental conditions described by Garret et al. (1999) and Xavier et al. (2014) were used with slight modifications. A homogeneous sample was obtained by crumbling and mixing three cupcakes of each fortification level. Then, 2 g of the cupcake sample was mixed with 5 mL of  $\alpha$ -amylase (200 U/mL) in  $\text{CaCl}_2$  (3.4 mM) for 1 min in a vortex and then 20 mL of 0.05% pepsin solution in 0.1 M HCl (pH 2.2) were added. The resulting mixture was incubated for 2 h under magnetic stirring in a water bath at 37 °C to complete the gastric phase of the *in vitro* digestion. This partially digested sample was quickly cooled in ice-water, pH adjusted to 7.0 with a 5% NaOH solution, mixed with 30 mL of a 0.3% bile extract in saline solution (3 M NaCl and 75 mM  $\text{CaCl}_2$ , pH 6.2), and incubated at 37 °C with magnetic stirring for 30 min. The mixture was then cooled again and mixed with 40 mL of a saline solution (0.1 M  $\text{NaHCO}_3$ , pH 8.4) containing 0.4% pancreatin and 0.07% lipase and incubated for a third time at 37 °C with magnetic stirring for 2 h. The aqueous phase was isolated from digested sample by centrifugation ( $12000\times g$ , 5 min, 4 °C) in an Avanti™ J-25 centrifuge (Beckman Coulter™, Brea, USA) equipped with a Beckman model JA 25.50 rotor (Kildare, Ireland). The aqueous phase was collected and used for measurement of lutein content and estimate the bioaccessibility efficiency (Granado-Lorencio et al., 2007).
- 2.5. Extraction and quantification of lutein in aqueous fraction. Lutein was extracted from the aqueous supernatants with diethyl ether and 10% NaCl aqueous solution. The mixture was spun at  $3000\times g$  for 5 min to enhance separation of water and organic layers. The latter was collected and the former extracted again to recover lutein remains, applying the same procedure. Combined organic extracts were dried in a rotary-evaporator and residue was dissolved in known volume of ethanol. The same procedure was also applied for extraction of lutein from cupcakes with the help of a homogenizer (Ultra-Turrax, model T-25, IKA

Labortechnik, Staufen, Germany) for 2 min at maximum speed. Absorbance of the final solution was measured at 445 nm in an HP-8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, USA). Lutein content was calculated applying Beer's Law with the extinction coefficient of lutein in ethanol  $E_{1cm}^{1\%} = 2550$  (Davies, 1976).

2.6.HPLC analyses: The profile of lutein and lutein esters was determined in a Jasco HPLC (Easton, USA) equipped with quaternary pump (model PU-2089-plus), autosampler (model AS-2055-plus) and diode array detector (MD-2010-plus). Chromatographic data were acquired and managed using the Jasco ChromPass Chromatography Data System software (version 1.8.6.1). For separation of lutein and lutein esters, a Luna column (Phenomenex, Torrance, USA) C18 (250×4 mm, 5 µm particle size). The mobile phase consisted on a linear gradient of acetone/water, from 75:25 (v/v) to 95:5 in 5 min, hold 95:5 for 7 min and to 100:0 in 3 min, maintaining this proportion for 10 min, and going back to initial conditions in 5 min. The UV-visible spectra were recorded in the 250-600 nm range, processing the chromatograms at 450 nm. Identification of lutein, *cis*-lutein and lutein esters was performed according to elution order on C18 column and characteristics of UV-visible spectrum, including  $\lambda_{max}$ , spectral fine structure (% III/II), and peak *cis* intensity (% $A_B/A_{II}$ ), in comparison with standards and data available in the literature (Britton, 1995).

2.7. Statistics. *In vitro* bioaccessibility of lutein (%) was determined as the ratio of lutein content in the aqueous fraction to lutein content per serving (65 g). The data were statistically analysed using Statistica software (Statistica 5.5, StatSoft, Tulsa, OK, USA). Results are expressed as mean values  $\pm$  standard deviation of three independent measurements, each one containing 3 replicates. Data were parametrically analysed and statistical comparison for significant differences for effects was performed by ANOVA. The significance level was set up at  $P < 0.05$ . Means were compared using the Tukey's test and significant at  $P < 0.05$ .

### 3. Results and Discussion.

Food fortification is a valuable strategy to help consumers meet their dietary needs. However, fortification may not materialize as one single tool to that purpose, and other approaches as the dietary diversification and supplementation may also coexist. This means

that although food fortification provides a relatively easy solution to reach micronutrient intakes at population, traditional dietary patterns and food diversification should be maintained (American Dietetic Association, 2001; Serra-Majem, 2001). Consequently, fortification levels were designed in this experiment to provide a substantial amount of lutein in one serving (1 cupcake of 65 g) to complement the intake of lutein from other food sources, to reach the daily 6 mg target figure established as the target amount for reduction of AMD risk (Landrum et al., 1997). Thus, the fortification range from 1 to 4 mg of lutein per serving would provide between 17-67% of the required lutein amount and this could be considered as the rational strategy for implementation of lutein fortification in wheat-based products. The experiments with higher fortification levels (6 mg and 12 mg per serving) were included to expand the knowledge about the effect of carotenoid amount in the food matrix in the bioaccessibility efficiency. Some ingredients of the cupcake receipt contain certain amounts of lutein (Handelman et al., 1999), and it is known that the baking process may reduce the initial carotenoid content, so that the first request was to determine the carotenoid content in the cupcakes with different fortification levels (including the unfortified control).

Table 1 shows the concentration of lutein in  $\mu\text{g}$  per g of serving before and after baking. The cupcakes of the control formulation present 2.99  $\mu\text{g}$  of lutein per g of serving before baking, an amount that did not decrease significantly after baking (2.85  $\mu\text{g}/\text{g}$ ) and that contribute with only 3% to the target daily 6 mg value (considering one serving = 65 g), although its bioaccessibility efficiency was also measured. It is meaningful that the amounts of lutein did not significantly decrease after baking at the higher fortification levels. Thus, the percentage of decrease was lower than 7% at the higher levels of lutein fortification (2-12 mg of lutein per serving) while decrease reached significant values at 0-1 mg of lutein per serving. These results are disagreeing with previous studies where a significant decrease of lutein content ranging 37-41% was observed (Abdel-Aal et al., 2010), but they correspond with other published data (Hidalgo et al., 2010; Read et al., 2015) that showed a limited extent of carotenoid degradation during cooking of wheat-based products. In those studies, the authors followed a different fortification strategy to reach the target amounts of lutein. Flours with a high carotenoid content were used to substitute the wheat flour and increase the lutein content in the final product, while in this study we performed the fortification with the addition of a lutein formulation. Therefore, effect of the baking process (heat) on carotenoid degradation seems to be noteworthy when the pigments form part of the microstructure of the flour matrix where other collateral degradation reactions may take place, including

enzymatic-based processes (Eyoun et al., 2003). Nevertheless, the heating conditions (time and temperature) and other degradative courses did not substantially affect the lutein content when the pigment is incorporated as an ingredient of the recipe.

Figure 1 depicts the bioaccessibility efficiency of lutein from cupcakes at different fortification levels. The control cupcake shows *ca.* 36% of lutein transfer from the product to the aqueous phase and at the following fortification levels this percentage shows an irregular behaviour with the increasing of the lutein content in the product. Thus, although the efficiency percentage rises significantly at 0.5 and 1 mg lutein/serving, 45% and *ca.* 65%, respectively ( $P < 0.05$ ), the bioaccessibility of lutein to the aqueous phase becomes stable at the next two fortification levels (2 and 3 mg lutein/serving) with 61% and *ca.* 58% of lutein transfer to aqueous phase, values not significantly different from the observed at 1 mg lutein/serving. At the next fortification levels the bioaccessibility efficiency significantly increases reaching the greater value observed in this experiment (81% at 4 mg lutein/serving), to decrease again to the previous plateau levels (60% at 6 mg lutein/serving). At the highest fortification level, lutein bioaccessibility increases again to 77% (12 mg lutein/serving), but this value is not significantly different from the top one. Despite this irregular behaviour, it seems *a priori* that the bioaccessibility efficiency of lutein tends to increase with the fortification amount. However, the linear correlation between both variables does not reach a good correlation level ( $R^2_{\text{adj}} = 0.43$ ). Indeed, if we consider the ratio between the point to point increase on the bioaccessible amount of lutein per g of serving to the corresponding increase on the lutein amount per g of serving, this is lower than 1. Thus, this means that the increments on lutein content per serving do not result in a net increase on lutein bioaccessibility. Nevertheless, these results agree with previous studies regarding carotenoid bioaccessibility from baked products (Kean et al., 2008; Read et al., 2015).

Among the factors determining the bioaccessibility efficiency of carotenoids, fat amount during digestion is considered the key one (Fernández-García et al., 2007). When the amount of lutein increased in the cupcake recipe, this one was not reformulated per the increased lutein levels with the addition of some fat source to improve subsequent digestion and bioaccessibility efficiency. Although cupcakes are low-fat products, the addition of some amounts of fat could be an alternative for improving the bioaccessibility levels of lutein in fortified wheat-based products.

Efficiency of the hydrolysis of lutein esters after digestion of fortified cupcake samples was measured because this is a significant factor in the subsequent steps for lutein absorption, as only free xanthophylls are detected in human plasma. Therefore, enzymatic hydrolysis

should take place either during digestion or within epithelial cells as pointed by Chitchumroonchokchai and Failla (2006). Figure 2 shows the chromatograms corresponding to the analyses of lutein in cupcake sample before digestion and to the lutein content in the aqueous phase after the digestion. As can be observed in the Figure 2b, most of the lutein content remains as esters, while a 30% has been hydrolysed to mono-esters or free lutein by the pancreatic lipase. That percentage value is below the range results obtained previously by Xavier et al. (2014) for a different food matrix. In this study, the same lutein formulation was applied to fortify the xanthophyll content in milk products.. Results support the low affinity of the pancreatic lipase over lutein esters, although the persistence to hydrolysis did not hindrance the transfer of the lutein esters to the aqueous phase that will be later available to the epithelial cells of the small intestine. This fact supports the possibility that other mucosal hydrolytic enzymes may hydrolyse those esters as mentioned before.

#### 4. Conclusions.

The addition of water-soluble formulation of lutein as an ingredient in the cupcake recipe to fortified the amounts of this xanthophyll in the final product showed satisfactory stability degree and resistance to the baking process. Degradation behaviour of the pigment is different when it does not form part of the microstructural flour matrix. The recipe also showed more than adequate bioaccessible efficiency of lutein according to the *in vitro* digestion procedure. These results point to new functional foods that may help to diminish the risk of degenerative processes related with deficient lutein intake/incorporation in humans. Further research should include the application of dynamic *in vitro* digestion model to perform more accurate estimations of carotenoid bioaccessibility according to critical factors like carotenoid and fat contents in the formulation, which are related to the efficiency of bioaccessibility.

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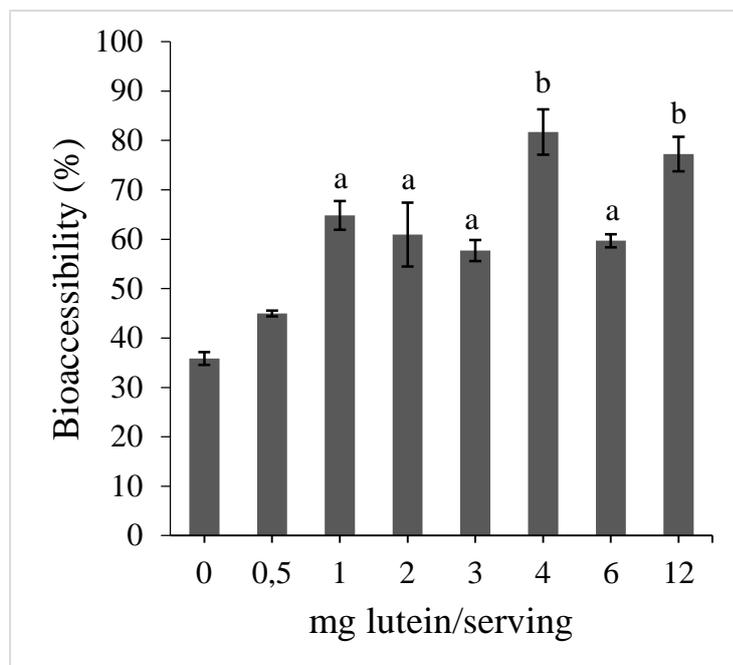
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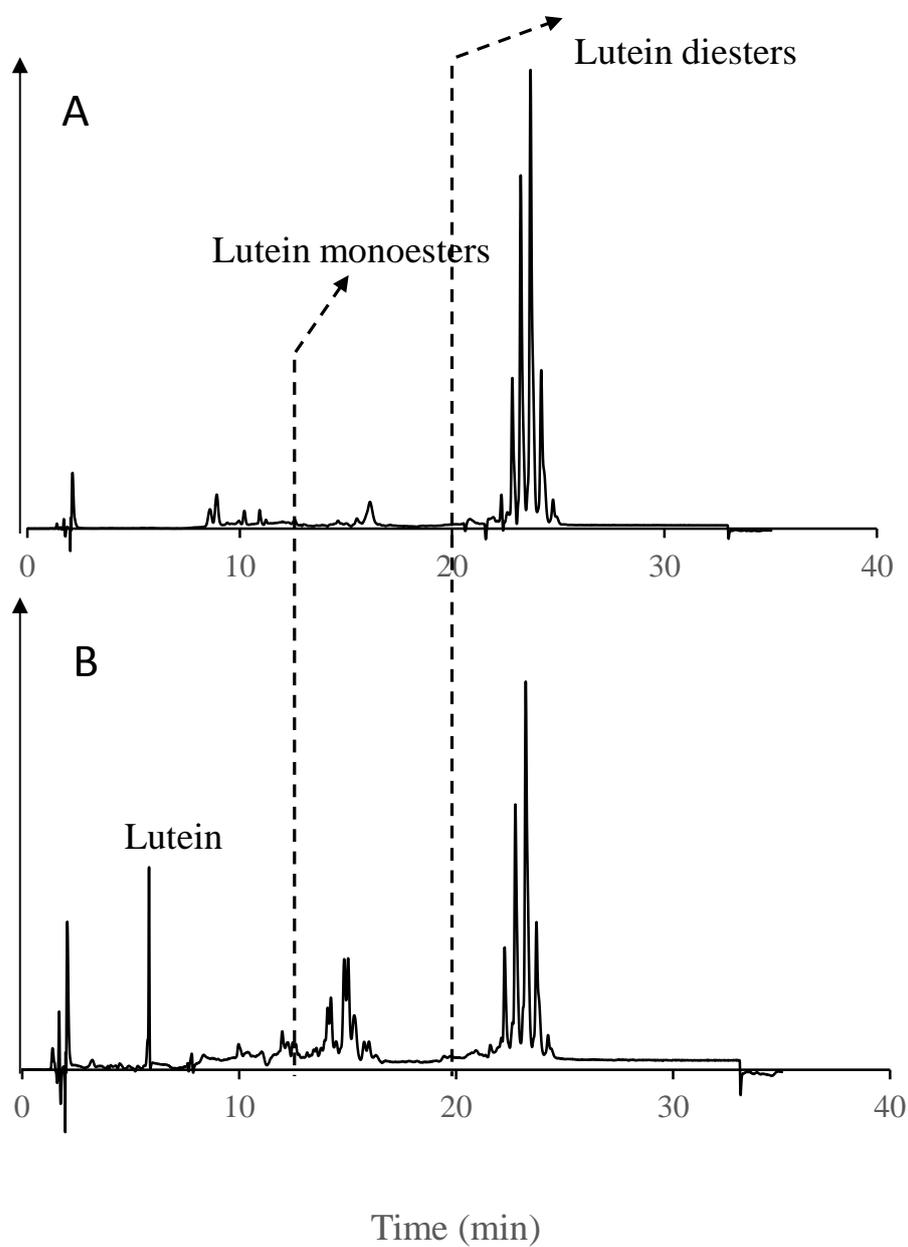
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**Figure 1.** Efficiency of bioaccessibility of lutein after *in vitro* digestion of cupcakes with different fortification levels. Data (%) are the mean values and the bars show the standard deviation (n=3). The values labelled with the same letter are not significantly different (ANOVA,  $P>0.05$ )



**Figure 2.** Chromatograms (extracted at 450 nm) corresponding to the analysis of lutein content in cupcake samples (A) and in the aqueous phase after *in vitro* digestion (B).



**Table 1.** Lutein content of cupcakes at different fortification levels before (pre) and after (post) baking process.

Sample <sup>a</sup>	Pre-baking ( $\mu\text{g/g}$ )*	Post-baking ( $\mu\text{g/g}$ )* <sup>b</sup>
0	$2.99 \pm 0.03$	$1.99 \pm 0.05$ (33.8)
0.5	$9.76 \pm 0.19$	$7.46 \pm 0.98$ (23.6)
1	$17.36 \pm 1.29$	$14.92 \pm 1.95$ (14.1)
2	$31.63 \pm 2.29$	$29.85 \pm 3.75$ (5.63)
3	$55.49 \pm 5.28$	$54.78 \pm 6.39$ (1.28)
4	$60.74 \pm 1.03$	$59.70 \pm 2.18$ (1.71)
6	$96.02 \pm 3.03$	$89.75 \pm 4.67$ (6.53)
12	$181.75 \pm 8.56$	$179.10 \pm 9.14$ (1.46)

\*Results are presented as  $\mu\text{g/g}$  of fresh weight. Data are mean  $\pm$  standard deviation (n=3).

<sup>a</sup>Fortification levels expressed as mg of lutein per serving.

<sup>b</sup>Numbers in parenthesis represent the decrease of lutein content in %.