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Digestive Stability, Micellarization, and Uptake by Caco-2 Human Intestinal Cell of Chlorophyll Derivatives from Different Preparations of Pea (*Pisum sativum* L.)

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The digestive stability, efficiency of micellarization, and cellular accumulation of the chlorophyll pigments of different preparations of pea were investigated, using an in vitro digestion procedure coupled with human intestinal Caco-2 cells. Fresh pea (FP), cooked fresh pea (CFP), frozen pea (FZP), cooked frozen pea (CFZP), and canned pea (CP) were subjected to simulated digestion. Although after digestion the pigment profile was modified for all samples, except CP, allomerization reactions and greater destruction of chlorophylls were observed in only FP, which should be due to enzymes in FP that were denaturalized in the rest of the test foods. A pigment extract of CFZP was also subjected to in vitro digestion, showing a positive effect of the food matrix on the pigment digestive stability. The transfer of the chlorophyll pigments from the digesta to the micellar fraction was significantly more efficient in CFZP (57%, $p < 0.0001$), not significantly ($p > 0.05$) different between CFP, FZP, and CP (28−35%), and lowest in FP (20%). Pheophorbide *a* stood out as the most-micellarized chlorophyll derivative in all of the samples, reaching levels of up to 98%. Incubation of Caco-2 cells with micellar fractions at the same concentration prepared from each test food showed that pigment absorption was considerably lower ($p < 0.006$) in cells incubated with FP, whereas there were no differences among the rest of the preparations. Therefore, factors associated with the food matrix could inhibit or mediate the chlorophyll pigment absorption. These results demonstrated that the industrial preservation processes of pea—freezing and canning—as well as the cooking have a positive effect on the bioaccessibility and bioavailability of the chlorophyll pigments with respect to the FP sample, emphasizing CFZP with greater bioaccesibilty degree.

KEYWORDS: Chlorophyll derivatives; in vitro digestion; Caco-2 cells; pea

INTRODUCTION

Epidemiological studies have consistently shown that the consumption of diets rich in fruits and vegetables is associated with a reduced risk of developing several chronic diseases such as cardiovascular disease and cancer. This evidence has made use of important efforts in identifying the vegetal food components with biological activity that, although not being essential nutrients for the life, promote in our organism a greater protection against the implantation of certain pathologies. Within this group of compounds, generically referred to as phytochemicals, the chlorophyll pigments stand out. Some reviews of the anticarcinogenic activity of chlorophyll compounds can be found in the literature (*1, 2*).

Although there is evidence that chlorophyll derivatives can be absorbed in humans, the information found on their bioaccessibility and bioavailability is limited. The term bioaccessibility for lipophilic food constituents is defined as the amount of a compound ingested that is transferred during digestion from the food matrix to the micelles (*3*), thus becoming accessible for its absorption in the intestinal tract (*4*). Bioavailability refers to the fraction of a compound that is absorbed from the intestine and becomes available for its use, metabolism, and/or storage by the organism (*2*).

It is probable that the general mechanism of chlorophyll absorption follows pathways similar to those taken by other xenobiotic compounds that require consideration of (a) efficient release of the chlorophylls from the food matrix, (b) stability to gastric and small intestinal digestive conditions, (c) solubilization of lipophilic derivatives, (d) uptake by small intestinal absorptive epithelial cells, and (e) secretion into circulation (*2*).

Compared with carotenoids or the other phytochemicals present in the normal diet that have a relatively low chemical reactivity, chlorophylls are very susceptible to experience

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modifications during digestion, particularly in four parts of the molecular structure: the chelate, the ester bond of the phytol alcohol (C-17[3]), the isocyclic ring (C-13[2]), and the basic porphyrin structure. In studies carried out with spinach puree, Ferruzzi et al. (*5*) demonstrated that native chlorophylls are transformed into Mg-free derivatives during the process of digestion and that the chlorophyll derivatives are absorbed by human intestinal cells. The same authors (*6*) studied the digestive stability and accumulation by human Caco-2 intestinal cells of the compound sodium copper chlorophyllin (SCC), which is a mixture of water-soluble chlorophyll derivatives, used as both colorant and diet supplement.

The bioaccessibility of a compound depends mainly on the physical properties of the food matrix. In the case of the carotenoids, it is established that they are released from edible plants only when the plant cell is ruptured, and this occurs only during the preparation of the processed foodstuff and/or mastication, and not during digestion. After release from the food matrix, the main limiting factor is the solubility of the carotenoid in the digesta (*7*). Considering the close association of chlorophylls with carotenoid pigments and their similar lipophilic character, it seems logical that the patterns of chlorophyll bioaccessibility and bioavailability are similar to those of the carotenoids.

The processing systems commonly used in the conservation of vegetable foods, and the cooking procedure, can modify the food matrix and, consequently, affect positively or negatively the bioaccessibility and bioavailability of the pigments present in the raw material. The aim of this research was to study the bioaccessibility and bioavailability of the chlorophyll pigments of different types of pea preparations that are commonly used for the consumer at home.

MATERIALS AND METHODS

All procedures were performed under green lighting to avoid any photooxidation of chlorophylls.

Chemicals. Sunflower oil was purchased at a local supermarket. Pepsin (porcine, 370 units/mg of solid), bile extract (porcine), pancreatin (porcine), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), sodium taurocholate, sodium bicarbonate, and potassium phosphate were provided by Sigma-Aldrich Chemical Co. (Madrid, Spain). Fetal bovine serum, penicillin−streptomycin−glutamine, nonessential amino acids, Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were purchased at Gibco (Invitrogen Ltd., Paisley, U.K.). Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands), HPLC reagent grade solvents were purchased at Teknokroma (Barcelona, Spain), and analysis grade solvents were supplied by Panreac (Barcelona, Spain). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA).

Preparation of Test Foods. Fresh peas (FP), frozen peas (FZP), and canned peas (CP) were purchased at a local market. FP and FZP were divided in two equal portions. One portion of either was retained raw, and the other was submitted to cooking as follow. The pea samples were added to boiling water in a covered stainless steel pot (1:3 food/water) and cooked on a moderate flame for 15 min. Then, samples were cooled to room temperature. The corresponding samples were referred to as cooked fresh peas (CFP) and cooked frozen peas (CFZP), respectively. Each pea sample (FP, CFP, FZP, CFZP, and CP) was cleaned with distilled water and wiped dry. Then, test foods were prepared by mixing equal weights of peas with saline solution (140 mM NaCl, 5 mM KCl) including 150 μM BHT and homogenizing to a pureed consistency with a polytron homogenizer. Aliquots of 10 g (corresponding to 5 g of pea and 5 g of saline solution) were weighed in 15 mL screw-capped tubes and were stored in a nitrogen atmosphere at −20 °C until used for the in vitro digestion test.

To obtain the pigment extract from the pea sample, an aliquot (10 g) of the pea puree was triturated repeatedly with acetone saturated with $MgCO_3$ and transferred to ethyl ether as described by Mínguez-Mosquera et al. (*8*). The ether phase was filtered through a bed of anhydrous Na_2SO_4 to remove the water completely and was evaporated in a rotavapor. The dry residue was stored in a nitrogen atmosphere at −20 °C until used for the in vitro digestion test.

In Vitro Digestion. The in vitro gastrointestinal digestion protocol used in this study was a combination of the methods proposed by Garret et al. (*9*) and Liu et al. (*10*). Before being subjected to the simulated digestions, the frozen aliquots of homogenized test food were thawed and mixed with 200 μL of sunflower oil to provide 4% fat based on pea weight (5 g) in the test meal. Then, the food sample was mixed with 13 mL of saline solution acidified to pH 2 with 0.1 M HCl and with the addition of pepsin porcine (4 mg/mL). The mixture was then readjusted for pH 2 with 1 M HCl (1 mL) and incubated in a thermostated water bath at 37 °C for 1 h with magnetic stirring at 500 rpm (gastric digestion). The intestinal phase was initiated by neutralization with 0.9 M $NaHCO_3$ (2 mL), addition of 6.5 mL of solution containing porcine pancreatin (2 mg/mL) and porcine bile extract (12 mg/mL) in 0.1 M $NaHCO_3$, and incubation in a thermostated water bath at 37 °C for 2 h with magnetic stirring at 500 rpm. Upon completion of the phases, aliquots (2 × 2 mL) of the digesta (D) were separated for pigment analysis. The rest was centrifuged at 48000*g* at 4 °C for 10 min to separate the solid residue, and the liquid was ultracentrifugated at 167000*g* at 4 °C for 40 min (Ti 50 rotor, Beckman model L7-65 Ultracentrifuge) to separate the aqueous micellar fraction (AMF) from the remaining solid residue and oil droplets. AMF was collected from the ultracentrifuge tubes with a Pasteur pipet (230 mm length) and was filtered (0.2 μm pore size) to remove contaminating aggregates. Aliquots (2 × 3 mL) were separated for pigment analysis. Samples were blanketed with nitrogen and stored at −20 °C until analysis.

In the trial of the pigment extract, some modifications were necessary to have similar conditions to the pea puree in the digestion process, as follows. Initially, deionized water (5 mL) and saline solution (5 mL) were added to achieve the pea puree volume. To get pH 2 in the gastric digestion, it was only necessary to add 0.05 mL of 1 M HCl, and then 0.95 mL of deionized water was added to adjust the volume to 1 mL. In the same way, for the intestinal phase, it was only necessary to add 0.5 mL of 0.9 M $NaHCO_3$, and 1.5 mL of deionized water was added to adjust the volume to 2 mL. Later, upon completion of the phases, aliquots (2 × 2 mL) of D were separated for pigment analysis, and the rest was directly ultracentrifuged under the same conditions as above.

Cell Culture. Caco-2 (colon adenocarcinoma) cells were obtained from the cell bank held at Centro de Investigaciones Biológicas (CIB-CSIC, Madrid, Spain). Cells were cultured in medium comprising high-glucose DMEM (pH 7.4), containing 4.5 g/L glucose and supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (0.292 mg/mL), nonessential amino acids (10 mL/L of a 100× stock solution), 2 mM glutamine, HEPES buffer (10 mmol/L), and 10% (v/v) heat-inactivated fetal bovine serum. Cells were seeded, in 75 cm^2 flasks with Nunclon-treated surface (Nunc A/S), at densities of 1 × 10^4 cells/cm^2, and incubated at 37 °C in a humidified atmosphere of air/carbon dioxide (95:5, v/v). The elevated carbon dioxide concentrations helped to maintain the pH of the medium (7.4). Medium was replaced every 2−3 days. Confluent cultures were achieved 7 days after seeding, and cultures were used for experiments 14 days after reaching confluency. Prior to the chlorophyll absorption experiments, the last two medium changes were carried out using serum-free medium.

Uptake of Micellar Chlorophyll Derivatives by Intestinal Cells. Assays of absorption by Caco-2 cell monolayers were performed with the analytical conditions perfected by Garret et al. (*9*). The potential cytotoxicity of the micellar fractions on cultures was evaluated in pilot studies. Gross morphologic appearance, the number of domes per microscopic field, and the protein content per flask were similar in differentiated cultures incubated in medium with and without micellar fraction for as long as 12 h. This demonstrated that exposure to micelles did not adversely affect cellular integrity.

The aqueous micellar fractions obtained from the in vitro digestion process were filtered and appropriately diluted with saline solution to obtain the same concentration in the cell medium (0.2 μM) for all test

Table 1. Profile and Content (Nanomoles per Gram of Pea) of Chlorophyll Pigments Depending on the Type of Pea Processing[a]

	fresh pea		frozen pea		
chlorophyll pigment[b]		cooked		cooked	canned pea
chl *a*[c]	110.35 ± 4.75	56.35 ± 7.35	120.42 ± 3.35	77.77 ± 6.97	
chld *a*			0.64 ± 0.51	1.45 ± 0.01	
AD chl *a*	4.13 ± 0.56	4.53 ± 0.19	6.63 ± 1.57	5.77 ± 0.76	
phy *a*[c]	1.68 ± 0.17	30.71 ± 3.68	13.53 ± 1.66	42.22 ± 5.32	41.53 ± 1.49
pho *a*		0.13 ± 0.08	0.17 ± 0.10	1.88 ± 0.09	3.13 ± 0.16
AD phy *a*					0.12 ± 0.07
pyro-phy *a*		0.06 ± 0.02		0.87 ± 0.03	69.86 ± 2.26
pyro-pho *a*					1.15 ± 0.04
15-G-phy *a*					1.46 ± 0.13
chl *b*[c]	34.16 ± 0.28	18.52 ± 1.93	39.99 ± 1.70	34.24 ± 0.86	
chld *b*			0.04 ± 0.02	0.07 ± 0.02	
AD chl *b*	0.32 ± 0.08	2.46 ± 0.51	1.96 ± 0.04	1.29 ± 0.13	
phy *b*[c]	0.19 ± 0.04	5.68 ± 0.77	1.49 ± 0.38	7.37 ± 0.92	16.57 ± 0.88
pho *b*		0.05 ± 0.02	0.15 ± 0.02	0.20 ± 0.03	0.53 ± 0.05
AD phy *b*					0.62 ± 0.19
pyro-phy *b*					24.78 ± 1.14
15-G-phy *b*					1.97 ± 0.17
total series *a*	116.16 ± 0.42	91.79 ± 3.24	141.39 ± 4.05	129.97 ± 0.78	117.25 ± 4.23
total series *b*	34.68 ± 0.12	26.71 ± 0.94	43.63 ± 2.27	43.17 ± 0.24	44.47 ± 1.98
total chls	150.84 ± 0.54	118.49 ± 4.18	185.02 ± 6.31	173.14 ± 0.54	161.73 ± 6.16

[a] Data represent the mean value ± SE ($n = 3$). [b] Abbreviations: chl, chlorophyll; chld, chlorophyllide; AD, allomerized derivatives; phy, pheophytin; pho, pheophorbide; G, glyoxylic acid. [c] Data include the respective epimer.

foods. The diluted micellar fractions were mixed with basal DMEM in a 1:3 (v/v) ratio (test medium), 25 mL was added to each monolayer, and cultures were incubated at 37 °C for 5 h. Eventually, the medium was removed and the monolayers, were washed two times with 2 mL of PBS containing 5 mmol/L sodium taurocholate, with the aim of removing pigments adhered to cell surfaces. Finally, cells were scraped into 4 mL of ice-cold phosphate-buffered saline containing 10% (v/v) ethanol. Samples were overlaid with nitrogen and stored at −20 °C for a maximum of 2 days.

Extraction and Analysis of Chlorophyll Pigments. Chlorophyll pigments were extracted from pea samples (5 g) with acetone as described by Mínguez-Mosquera et al. (*8*). In the case of the digesta and micellar fraction, the frozen aliquots were thawed, and the chlorophyll pigments were extracted by addition of 2 mL of acetone (0.2% BHT), 2 mL of diethyl ether, and 2 mL of 10% NaCl and vortexed for 1 min. The sample was then centrifuged at 4500*g* for 5 min to hasten phase separation. The diethyl ether layer was collected and saved. The extraction with diethyl ether was repeated a total of three times, and the combined diethyl ether fractions were dried under a stream of nitrogen and redissolved in 0.5 mL of acetone. An aliquot (50 μL) was analyzed by reverse-phase HPLC.

In the case of pigment extraction from Caco-2 cells, the frozen samples were thawed, and 1 mL of ethanol (0.1% BHT) and 2 mL of acetone (0.2% BHT) were added. Then the cell homogenates were sonicated for 5 min on ice. After this, 2 mL of diethyl ether and 2 mL of 10% NaCl were added and vortexed for 1 min, and the extraction process was continued as above. The dried residue was redissolved in 0.25 mL of acetone, and an aliquot of 50 μL was analyzed by reverse-phase HPLC.

HPLC analysis of chlorophyll pigments was performed according to the method described by Mínguez-Mosquera et al. (*11*), using a reverse phased column (20 × 0.46 cm) packed with 3 μm C_{18} Spherisorb ODS2 (Teknokroma, Barcelona, Spain) and an elution gradient with the solvents (A) water/ion-pair reagent/methanol (1:1:8, v/v/v) and (B) acetone/methanol (1:1 v/v), at a flow rate of 1.25 mL/min. The ion-pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The allomerized chlorophylls were identified by cochromatography with the corresponding standard and from their spectral characteristics as has been described in detail in previous papers (*12, 13*). The online UV−vis spectra were recorded from 350 to 800 nm with the photodiode array detector. Pigments were detected by absorbance at 410, 430, 450, and 666 nm and quantified from the corresponding calibrate curves.

Calculations and Statistical Analysis of Data. For each pea sample, analyses were performed in triplicate, and the chlorophyll pigment content was expressed in nanomoles per gram of pea. For studies characterizing the in vitro digestion procedure, 10 independent digestions were made for each test food, and each was analyzed in duplicate. Composition of chlorophyll pigments in digesta and in the starting raw material was expressed as percentage with respect to the total chlorophyll content. Percentage of pigment recovery in D was calculated as nanomoles in D × 100/nanomoles in starting raw material. Percentage of micellarization was calculated as nanomoles in AMF × 100/nanomoles in D. This gives information of which proportions of those pigments present in D are ready for the enterocyte absorption.

For cellular studies, the diluted aqueous fraction from each digesta was added to six replicate flasks with differentiated monolayers of Caco-2 cells. The uptake of chlorophyll derivatives by Caco-2 cells was expressed as percentage of absorption, and it was calculated as nanomoles of pigments added to the cell monolayer × 100/nanomoles accumulated by the cell monolayer.

Data were expressed as means ± SE. The data were analyzed for differences between means using one-way analysis of variance (ANOVA). Duncan's multiple-range test was used as a post hoc comparison of statistical significance (p values < 0.05). All statistical analyses were performed using Statistica for Windows (StatSoft, Inc., 2001).

Apparatus. Equipment included a pH-meter, model pH 555 (Teknokroma, Barcelona, Spain), an Ultra-Turrax, model T-25 polytron homogenizer (Janke Kunker, IKA-Laboratechnik), a Büchi rotavapor, model R 110 (Laboratoriums-technik AG), a centrifuge, model Avanti J-25, an ultracentrifuge, model L7-65 (Beckman Coulter, Palo Alto, CA), and an HP 1100 Hewlett-Packard (Palo Alto, CA) liquid chromatograph fitted with an HP 1100 automatic injector and diode array detector.

RESULTS AND DISCUSSION

Pigment Profile in the Test Foods. The qualitative and quantitative profile of the pigments present in the pea samples used in the study differed depending on the type of treatment the food had been subjected to (**Table 1**). As Edelenbos et al. (*14*) have already found in six cultivars of pea, the major components in the fresh peas (FP) were chlorophylls *a* and *b*, accompanied by their respective *a′* and *b′* epimers, with a minimum presence of the allomerized derivatives 13^2-OH-

Table 2. Quantitative Distribution (Percent) of Chlorophyll Pigments in the Test Foods Previous (pre-d) and Posterior (post-d) to in Vitro Digestion Process

	fresh pea				frozen pea				canned pea	
			cooked				cooked			
chlorophyll pigment[a]	pre-d[b]	post-d[c]	pre-d[b]	post-d[c]	pre-d[b]	post-d[c]	pre-d[b]	post-d[c]	pre-d[b]	post-d[c]
chl *a*[d]	73.16 ± 4.75		46.94 ± 3.68		65.08 ± 0.42		44.92 ± 4.02			
chld *a*					0.35 ± 0.07		0.84 ± 0.00			
AD chl *a*	2.74 ± 0.09	0.46 ± 0.03	3.77 ± 0.29		3.59 ± 0.69		3.33 ± 0.45			
phy *a*[d]	1.11 ± 0.08	64.92 ± 0.78	25.58 ± 1.02	68.56 ± 0.37	7.31 ± 0.65	68.60 ± 0.24	24.39 ± 3.18	68.29 ± 0.11	25.63 ± 0.18	25.37 ± 0.28
pho *a*			1.41 ± 0.07	1.65 ± 0.04	0.09 ± 0.01	0.61 ± 0.03	1.09 ± 0.06	1.64 ± 0.03	1.93 ± 0.06	1.22 ± 0.15
AD phy *a*		7.77 ± 0.68		4.09 ± 0.09		1.92 ± 0.15		1.38 ± 0.03	0.51 ± 0.04	0.74 ± 0.22
pyro-phy *a*		0.27 ± 0.02	0.05 ± 0.01	1.28 ± 0.32		0.32 ± 0.05	0.50 ± 0.02	0.79 ± 0.06	43.10 ± 0.40	44.95 ± 0.48
pyro-pho *a*									0.71 ± 0.02	0.43 ± 0.08
15-G-phy *a*									0.90 ± 0.06	1.19 ± 0.34
chl *b*[d]	22.65 ± 0.26		15.43 ± 0.65		21.61 ± 0.17		19.78 ± 0.41			
chld *b*					0.02 ± 0.00		0.04 ± 0.01			
AD chl *b*	0.21 ± 0.01	2.61 ± 0.22	2.05 ± 0.39	0.92 ± 0.08	1.06 ± 0.01	0.28 ± 0.02	0.75 ± 0.08			
phy *b*[d]	0.13 ± 0.06	18.64 ± 0.26	4.73 ± 0.18	21.18 ± 0.08	0.81 ± 0.18	25.72 ± 0.17	4.26 ± 0.55	25.62 ± 0.06	10.23 ± 0.20	9.55 ± 0.11
pho *b*			0.04 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.10 ± 0.01	0.12 ± 0.02	0.23 ± 0.01	0.33 ± 0.02	0.05 ± 0.01
AD phy *b*		5.34 ± 0.33		2.22 ± 0.06		2.10 ± 0.18		2.05 ± 0.07	0.38 ± 0.11	0.62 0.08
pyro-phy *b*									15.29 ± 0.44	14.87 ± 0.21
15-G-phy *b*									1.22 ± 0.08	1.00 ± 0.05
total series *a*	77.01 ± 0.77	73.41 ± 0.25	77.75 ± 0.26	75.58 ± 0.13	76.42 ± 0.43	71.80 ± 0.10	75.07 ± 0.25	72.10 ± 0.04	72.56 ± 0.30	73.90 ± 0.28
total series *b*	22.99 ± 0.77	26.59 ± 0.25	22.25 ± 0.26	24.42 ± 0.13	23.58 ± 0.42	28.20 ± 0.10	24.93 ± 0.25	27.90 ± 0.04	27.44 ± 0.30	26.10 ± 0.28
total AD	2.95 ± 0.28	16.18 ± 1.02	5.82 ± 0.69	7.23 ± 0.16	4.65 ± 0.99	4.30 ± 0.31	4.08 ± 0.6	3.43 ± 0.10	2.31 ± 0.21	1.84 ± 0.29

[a] Abbreviations: see **Table 1**. [b] Data represent the mean value ± SE ($n = 3$). [c] Data represent the mean value ± SE for 10 independent digestions and each analyzed in duplicate. [d] Data include the respective epimer.

chlorophylls *a* and *b* and of pheophytins *a* and *b*. However, in the rest of the preparations, which had been subjected to one or another heat treatment, the pigment profile was modified substantially.

In the frozen peas (FZP), the number of chlorophyll derivatives was increased, with chlorophyllides *a* and *b* and pheophorbides *a* and *b* being detected in very low amounts, and there was an increase in the proportion of pheophytins and the allomerized chlorophylls. This pigment profile was due to the transformation of the chlorophyll molecule during the industrial preparation of frozen peas. This process includes the prior blanching of the raw material by a heat treatment (80−100 °C) that activates the enzyme chlorophyllase, originating chlorophyllides, and subsequently pheophorbides due to the increasing free-acidity (*15*).

In the canned peas (CP), the pigment profile was totally different, as this preparation includes a prior process of sterilization that involves a heat treatment (around 121 °C), which is very drastic for the chlorophyll molecule, with complete transformation mainly to pheophytins and pyropheophytins. That is, besides the pheophytinization reaction, there is also oxidation on the isocyclic ring. Consequently, chlorophyll forms that include Mg in their structure (chlorophylls and chlorophyllides) were not present in CP and, besides pheophytins and pheophorbides, new derivatives were found, such as pyropheophytins *a* and *b* (the major forms), 13^2-OH-pheophytins *a* and *b*, pyropheophorbide *a*, and 15-glyoxylic acid pheophytins *a* and *b*. Chemically, these last are also considered to be allomerized derivatives, characterized by having the isocyclic ring of the chlorophyll molecule open, and are formed when oxidative reactions take place in acid or slightly alkaline medium (*12, 16*). This was the first time that 15-glyoxylic acid pheophytins *a* and *b* have been detected in processed peas, and they have been found previously only in table olives (*13, 17*).

In the samples of peas cooked in the laboratory—cooked fresh peas (CFP) and cooked frozen peas (CFZP)—the qualitative pigment profile was mostly the same as initially in the respective raw material obtained from the market, except for the detection of minimum amounts of pyropheophytin *a* and pheophorbides *a* and *b*. However, the pigment ratio was different, as the amount of chlorophylls was lower, essentially in favor of pheophytins. At the same time, there was some degradation of chlorophylls to colorless products during the preparation of these test foods.

Pigment Stability during Simulated Digestion. After the gastric and small intestinal in vitro digestion process of test foods, it was found that the pigment profile was modified for all of the samples, except CP (**Table 2**). The acid conditions, which govern in the gastric digestion phase, caused the complete disappearance of chlorophylls *a* and *b*, in favor of the respective Mg-free derivatives. In CP, the pigment profile was unaltered, because the transformations due to an acid pH had already occurred in the raw material.

During the digestive process, allomerization reactions were seen in sample FP, being increased the allomerized derivatives from some 3% (of the total chlorophyll compounds) in the raw material to some 16% after digestion. In the rest of the samples, there were no significant differences ($p > 0.05$) in the relative presence of allomerized derivatives before and after the process of gastric and intestinal digestion. Allomerization is a reaction involving the oxidation of $C\text{-}13^2$ of the chlorophyll molecule by triplet molecular oxygen and can take place by both enzymatic and chemical pathways (*18*). In the present study, if the reaction had been purely chemical, without the intervention of enzyme, there should also have been a perceptible allomerization reaction in the rest of the test foods that initially contained chlorophylls *a* and *b*, as the conditions of digestion were the same for all of them. The fact that allomerization was observed only in the digestion of FP indicated that this reaction was promoted by an enzyme (probably peroxidase), which in the other test foods was denaturalized due to the prior heat treatments to which the peas had been subjected for their processing. With regard to such a conclusion, we found, on the one hand, the studies of Gökmen et al. (*19*) noting that the blanching of peas at 80 °C for 2 min inactivates some 90% of the initial activity of peroxidase and, on the other, work carried out in olives demonstrating the presence in the fruits of a peroxidative activity implicated in the formation of allomerized

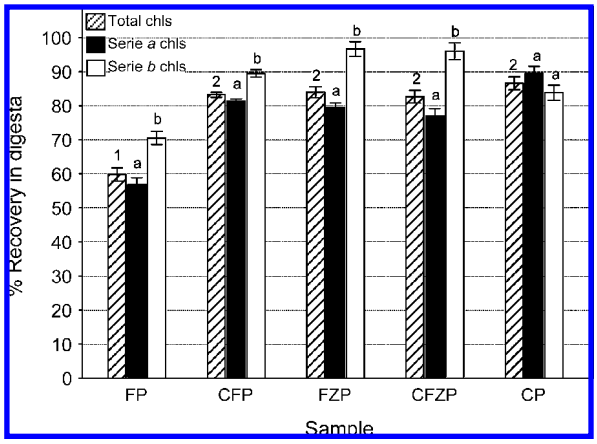

Figure 1. Percentage of total, series *a*, and series *b* chlorophylls (chls) recovered after simulated in vitro digestion of fresh pea (FP), cooked fresh pea (CFP), frozen pea (FZP), cooked frozen pea (CFZP), and canned pea (CP). Data represent the mean value ± SE for 10 independent digestions, each analyzed in duplicate. Different numbers above the error bars indicate significant differences of total chls between test foods ($p < 0.05$). Different letters above the error bars within the same test food indicate significant differences between series *a* and series *b* chls ($p < 0.05$).

chlorophyll derivatives, as intermediate products, leading to their final degradation to colorless catabolites (*20*).

Ferruzzi et al. (*5*) detected high levels of allomerized pheophytins after the digestion of fresh spinach puree, but not after the digestion of heat- and acid-treated spinach puree. Those authors suggest that, in the fresh spinach puree, the oxidation of chlorophyll is prior to the formation of pheophytins during digestion—hence the different levels of allomerization found between that sample and the acid- and heat-treated one, in which there are no chlorophylls, but pheophytins. We now add that, although the allomerization reaction might be more favored on the chlorophyll molecule than on pheophytin, the main cause of the differences found by those authors was due to the inactivation of enzymes in the spinach purees that have been subjected to a prior heat treatment.

To be able to determine whether the quantitative effect of digestion was equal on all of the food matrices used in the study, the percentage of chlorophyll pigments recovered in digesta, with respect to the initial content in the starting raw material, was calculated (**Figure 1**). The recovery of chlorophyll pigments after gastric and intestinal digestion was significantly lower ($p < 0.0001$) in FP (60%) than in the rest of the samples (83−87%), among which there were no significant differences. These results showed that the chlorophyll losses caused by heating the peas in boiling water for 15 min (**Table 1**) were balanced subsequently with a greater recovery of the pigments after the process of digestion. The greater destruction of chlorophylls during the digestion of FP suggested that in that test food were operating enzymatic systems able to produce the degradation of the chloroplast pigments to colorless products while, by the same reason as above, the enzymes were denaturalized in the rest of test foods. Although it could be expected that the extremely low pH conditions, which govern the gastric digestion phase, could denaturalize the enzyme, it is known that the tobacco peroxidase is stable at extremely low pH, showing a maximum catalytic activity at pH 1.8 (*21*).

Both peroxidase and lipoxygenase are enzymes that have been related with the loss of pigments during the storage of vegetables (*22, 23*) and which lose some 90% of their initial activity or their total activity, respectively, when peas are blanched at 80 °C (*19*). Characterization studies on peroxidase in two varieties of melon (*24*) and on lipoxygenase in green peas (*25, 26*) confirm that the conditions of temperature (37 °C) and pH (around 7 in the intestinal phase) that govern in digestion are ideal for the activity of these enzymes. Chisari et al. (*24*) found maximum enzymatic activity at 25 and 45 °C, depending on the variety of melon studied, and it remainsed high in the mid-temperature range in both cases. With regard to the pH conditions, although enzymatic activity is maximum at pH 4.5 for both varieties of melon, it continues to be quite high up to pH 7 (around 70% of the maximum value). On the other hand, Gökmen et al. (*25*), working with raw extract of pea, found maximum lipoxygenase activity at 30 °C, and at 37 °C the activity was still considerable, also around 70% of the maximum. With regard to pH, they found a range of high activity between 5 and 6.5 units, with a maximum at pH 6, and the activity was markedly lower at pH 7. However, Yoon and Klein (*26*) isolated four isoenzymes from peas, finding that one of the major ones, PL I, with chlorophyll-bleaching activity, had its maximum activity between pH 5 and 7.

Nevertheless, it was possible that the presence of active enzymes in FP was not the only cause of the differences found in pigment stability during digestion. With the aim of studying the effect of the food matrix on the transformation of chlorophylls during the in vitro digestion, a pigment extract from a sample of CFZP was subjected to this process. In that trial, the chlorophyll derivatives of both series *a* and *b* were recovered in lower proportion from the extract than when the digestion was performed with the complete food matrix, displaying a destruction of 48 and 18%, respectively, against the 17 and 3% with the corresponding test food. Thus, the greater digestive stability of the pigments in the processed peas could also be mediated by the food matrix, in the sense that the different processing systems could make them more accessible to components with antioxidant capacity, such as phenols, carotenoids, and ascorbic acid, that act as such during the digestion of the food, preventing oxidation of the chlorophylls. In accord with this hypothesis, there is a very recent study on the effect of different methods of cooking on the nutritional characteristics of various vegetables (*27*). The authors found an overall increase of antioxidant capacities in all of the cooked vegetables, which they attribute to softening of the matrix and an increased extractability of the compounds, which can convert them into more-antioxidant chemical species.

On distinguishing the two chlorophyll fractions in the same sample (**Figure 1**), the series *b* fraction (mainly pheophytin *b*) was more stable to digestion ($p < 0.0003$) than series *a* (mainly pheophytin *a*), with the exception of CP, in which pyropheophytins were the major chlorophyll derivatives in each fraction. The differences were more marked in the food matrices that had been frozen (FZP and CFZP). In CP there were no significant differences ($p > 0.05$) between the percentage of retention in digesta of series *a* and series *b*, because a higher recovery of the first was found. This could be due to a greater stability of pyropheophytin *a* than pheophytin *a* to the digestion conditions.

Micellarization of Pigments during Digestion. Although all of the pigments present in the digesta were transferred to a greater or lesser extent to the micellar aqueous fraction, for simplicity **Figure 2A** shows the percentage of transference only of the major compounds, pheophytin *a* and pheophytin *b*, and of pheophorbide *a*, the chlorophyll derivative that showed the highest bioavailability. For sample CP are also included pyropheophytins *a* and *b*, the major chlorophyll forms in that

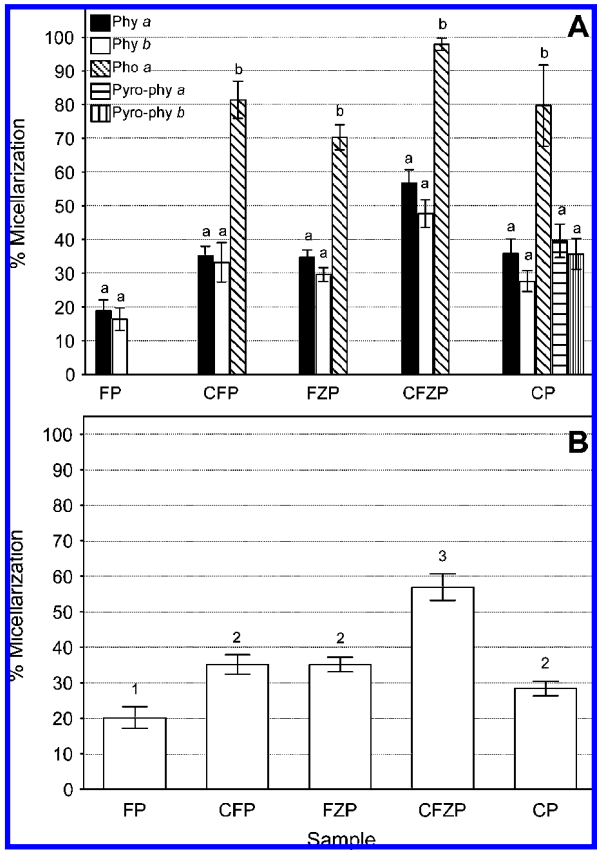

Figure 2. Percentage of transference from the digesta to the aqueous micellar fraction during simulated in vitro digestion of FP, CFP, FZP, CFZP, and CP of (**A**) pheophytin *a* (Phy *a*), pheophytin *b* (Phy *b*), pheophorbide *a* (Pho *a*), pyropheophytin *a* (Pyro-phy *a*), and pyropheophytin *b* (Pyro-phy *b*) and (**B**) total chlorophyll derivatives. See **Figure 1** for abbreviations. Data represent the mean value ± SE for 10 independent digestions, each analyzed in duplicate. For **A** different letters above the error bars within the same test food indicate significant differences between pigment micellarization ($p < 0.05$). For **B** the presence of different numbers above the error bar indicates significant differences between test foods in the micellarization of total chlorophyll derivatives ($p < 0.05$).

test food, but these are practically absent in the rest. The percentage of micellarization of the pigments varied depending on the test food used in the digestion. The most efficient ($p < 0.05$) transfer, for all pigments, was in CFZP. Comparison between pigments in the same matrix revealed that pheophorbide *a* stood out as the most-micellarized derivative in all of the samples, reaching levels of up to 98%, whereas within each test food, no significant differences were found between the degree of micellarization of pheophytin *a* and pheophytin *b* or, in the case of sample CP, between pyropheophytin *a* and pyropheophytin *b* and between pheophytins and pyropheophytins *a* and *b*.

An overall evaluation of the chlorophyll derivatives present in each digestate showed that the micellarization of the total chlorophylls was significantly more efficient in CFZP (57%, $p < 0.0001$) (**Figure 2B**). In CFP and FZP, the transfer of total pigments from the digestate to the micellar fraction was similar (35%), and although it was somewhat higher than that obtained with CP (28%), the difference was not significant ($p > 0.05$). The lowest percentage of micellarization was in FP (20%).

The chlorophylls are localized in the lipid matrix of the thylakoid membranes of the chloroplast, forming pigment−protein macromolecular complexes. An important requirement for the satisfactory use of the chloroplast pigments of plants is that the cellulose structure of the cell walls is ruptured, liberating the pigments into the luminary fluids of the intestine. This is helped by cooking and mastication during ingestion of the food (*28*). In the present study, we found that, in general, the degree of micellarization of the chlorophyll pigments was directly related with the treatment to which the raw material had been subjected. Thus, the preparation of peas involving a double heat treatment (CFZP—first a blanching before freezing and later the cooking in the laboratory) resulted in the highest degree of micellarization. A second range of micellarization was in those types of preparations involving a single heat treatment, such as CFP, FZP, and CP, all with a similar micellarization degree. The sample of pea subjected directly to digestion without prior treatment (FP) presented the lowest degree of micellarization.

In contrast to our results, in a study carried out on the degradation and absorption of carotenoids and chlorophylls during the digestion of fresh spinach puree, heat- and acid-treated spinach puree, and spinach puree treated with $ZnCl_2$, Ferruzzi et al. (*5*) obtained a lower degree of micellarization in the samples that had been previously processed than in the corresponding fresh spinach puree. The authors attributed this result to the effects of processing on the digestibility of the vegetable tissue during the thermal processing, acidification, or $ZnCl_2$ treatment, components can be formed or liberated that can impair digestion enzyme activities or alter micelle formation by interacting with the bile salts. For carotenoids and chlorophylls to be transferred from the foodstuff to the micelles, the presence of bile extract is necessary (*5, 9*). Dongowski (*29*) demonstrated that under in vitro conditions, the bile acids interact strongly with highly esterified pectins and that this interaction decreases with the degree of esterification. Sajjaanantakul et al. (*30*) concluded that a heat treatment in aqueous medium causes the de-esterification of the methyl groups present in the pectins. From the results of those authors, it was perfectly presumable that, in our experiment, the different processings to which the peas had been subjected before digestion caused some de-esterification of the pectins present in the peas, with a consequent decrease in the interaction with the bile salts (*29*), enabling the micellarization of chlorophylls.

Uptake of Micellarized Chlorophylls by Caco-2 Cells. In the studies carried out with spinach puree discussed in the previous section, Ferruzzi et al. (*5*) established that the amount of pigments absorbed by intestinal cells is proportional to the pigment content of the micellar fraction and emphasized the importance of an efficient micellarization for the maximization of cellular concentration of chlorophylls and carotenoids. However, although this is so, during the in vitro digestion process are present other compounds of the test foods, which may also affect the chlorophyll pigment absorption, as Reboul et al. (*31, 32*) showed for some dietary antioxidants, such as lutein and α-tocopherol. These authors suggested that the intestinal absorption of those compounds can be impaired by the presence of other dietary antioxidants, such as the carotenoids lycopene and β-carotene and the polyphenol naringenin. In the present study, we thought that the changes provoked in the food matrix by the processing systems of pea could cause differences in the composition of the micellar fraction that could have an effect on the absorption of the chlorophyll pigments. For verifying this point, the aqueous micellar fractions obtained from the in vitro digestion process were filtered and diluted with saline solution to obtain the same concentration in the cell medium for all test foods.

After 5 h of incubating the Caco-2 cells with the different micellar fractions, it was found that, in all cases, pheophytins *a*

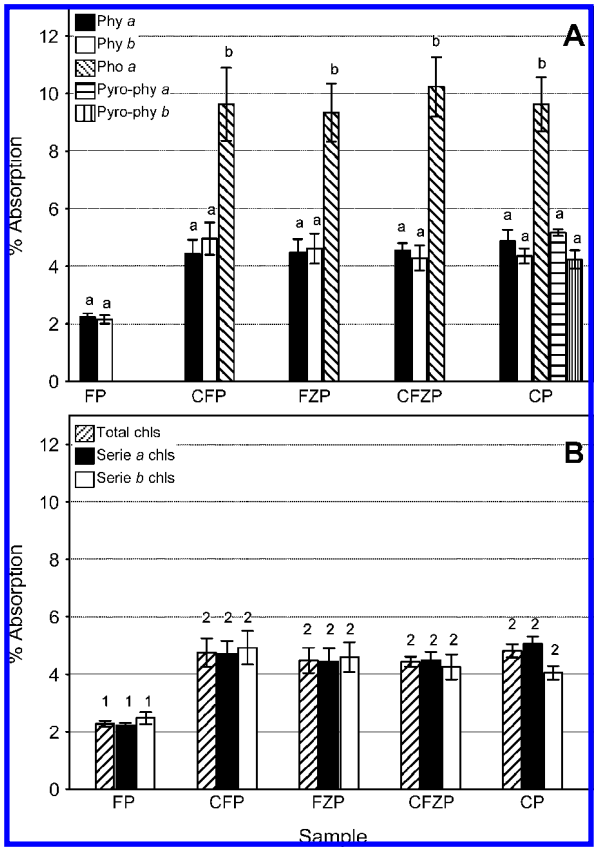

Figure 3. Uptake by Caco-2 cells (percentage of absorption) of micellarized chlorophyll pigments generated from the simulated in vitro digestion of FP, CFP, FZP, CFZP, and CP: (**A**) Phy *a*, Phy *b*, Pho *a*, Pyro-phy *a*, and Pyro-phy *b*; (**B**) total, series *a*, and series *b* chlorophylls. See **Figures 1** and **2** for abbreviations. Data represent the mean value ± SE for six samples. For **A** different letters above the error bars within the same test food indicate significant differences between pigment absorption ($p < 0.05$). For **B** the presence of different numbers above the error bar indicates significant differences between test foods, in the absorption of total, series *a*, and series *b* chlorophyll derivatives ($p < 0.05$).

and *b*, and the corresponding allomerized compounds, were captured from the respective micelles. The same happened with pheophorbide *a*, when it was present in the micelles, and pyropheophytins *a* and *b*, only in the test food CP, where they were the dominant pigments. The percentage of pigment absorbed with each test food is shown in **Figure 3A**. Pheophorbide *a* stood out as the pigment absorbed in the greatest amount for all the tested foods in which it was present. No significant differences ($p > 0.05$) were found in the percentage of absorption for the rest of the pigments in the same sample.

Figure 3B compares the total of chlorophyll derivatives of both series *a* and *b*, as well as the total chlorophyll pigments, absorbed by the Caco-2 cells from the different micellar fractions. It can be seen that although, after adjustment with saline solution, the concentration of total chlorophyll pigments in the aqueous micellar fraction was the same for all of the tested foods, pigment absorption was considerably lower ($p < 0.006$) in the cells incubated with the micellar fraction of FP, whereas there were no differences among the rest of preparations, which had all been subjected to some form of heat treatment.

The results obtained in the present study demonstrated that the micellarization degree of the pigments was not the only factor intervening in the amount of pigments absorbed and that other factors associated with the food matrix could inhibit or mediate in their absorption. It is known that heat treatments, to which foodstuffs are usually subjected, modify their chemical composition from the original state (*27*). According to this, the higher chlorophyll pigment absorption obtained with the processed pea samples (CFP, FZP, CFZP, and CP) suggested that the composition of the corresponding micellar fractions should be different with respect to the FP sample, having such difference a positive effect on the absorption of the chlorophyll derivatives.

ABBREVIATIONS USED

FP, fresh pea; CFP, cooked fresh pea; FZP, frozen pea; CFZP, cooked frozen pea; CP, canned pea; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; BHT, butylated hydroxytoluene; D, digesta; AMF, aqueous micellar fraction.

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