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ARTICLE

Influence of the Chlorophyll Pigment Structure on Its Transfer from an Oily Food Matrix to Intestinal Epithelium Cells

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Chlorophyll *a*, chlorophyll *b*, and the Mg-free chlorophyll derivatives pheophytin *a*, pheophytin *b*, pyropheophytin *a*, pheophorbide *a*, and pyropheophorbide *a*, dissolved in an oily matrix, were subjected to a simulated in vitro digestion procedure coupled with uptake by human intestinal Caco-2 cells. The native chlorophylls showed greater instability to the digestive process than the Mg-free chlorophyll derivatives. In addition to pheophytinization reactions, allomerization and oxidation to uncolored compounds were found to greater extents for the former. After digestion, the pigment dispersion degree in the colloid system (aqueous-"micellar" phase) showed significant differences ($p < 0.05$) among series *a* and series *b* derivatives. However, when a mixture of pheophytin *a* and pheophytin *b* was digested, there was a positive effect for pheophytin *b*. Both the dispersion degree and the accumulation rate by the Caco-2 intestinal epithelial cells were significantly higher ($p < 0.05$) for dephytylated chlorophyll derivatives. Differences in the transport route were also found. Whereas phytylated chlorophyll derivatives showed passive absorption by simple diffusion, the dephytylated ones showed passive absorption by facilitated diffusion in the lower range of concentrations tested. These results showed that the structural modifications of the chlorophyll pigments, mainly the de-esterification of phytol, significantly increased—by an estimated 65-fold—its transfer from the food matrix to the intestinal epithelial cells during digestion, making it more bioaccessible. The possible relationship between the phototoxicity associated with pheophorbide and the high bioaccessibility demonstrated in this work is discussed.

KEYWORDS: Chlorophyll; pheophytin; pyropheophytin; pheophorbide; pyropheophorbide; in vitro digestion; bioaccesibility; Caco-2 cells

INTRODUCTION

Epidemiological studies relating the consumption of fruits and vegetables with a reduction in cell aging and a lessened risk of developing certain forms of cancer have stimulated interest in plant food phytochemicals as physiologically active dietary components. Among the long list of bioactive compounds present in diets rich in green vegetables, chlorophyll pigments have been traditionally overlooked compared with other phytochemicals, despite being found in greater concentration than other widely studied phytochemicals. It has been demonstrated, in both in vitro and in vivo animal model assays, that the chlorophyll pigments exhibit a series of biological properties, such as antioxidant and antimutagenic activities, modulation of xenobiotic enzyme activity, and induction of apoptotic events in cancer cell lines, all consistent with the prevention of degenerative diseases. Despite this, the information available on their bioaccessibility and bioavailability is minimal (*1*).

The general assumption that chlorophyll compounds were not absorbed by humans led to the consideration that their physiological role was limited exclusively to intercepting mutagenic compounds in the gastrointestinal tract (*2*). However, recent research results have shown that these compounds are effectively absorbed by humans. First, chlorins were identified in the plasma of volunteers taking part in a study of chemoprevention with chlorophyllin (*3*), and subsequently the absorption of pheophytins has been demonstrated in a model system of human Caco-2 epithelial cells (*4*). This evidence has nourished very promising expectations of research aimed at elucidating the factors involved in the potential bioaccessibility and intestinal absorption of these bioactive compounds. Recently, it has been demonstrated that the industrial preservation processes of pea—freezing and canning—along with the cooking have a positive effect on the bioaccessibility of chlorophyll pigments (*5*).

The distribution and content of chlorophylls in the green tissues of fruits and vegetables depend on several factors, including species, variety, agro-climatic conditions, and postharvest treatments. Moreover, in comparison with other dietary phytochemicals having a relatively low chemical reactivity, the chlorophylls, outside their natural environment, are very sensitive to factors such as changes in pH or temperature, enzyme action, oxidation by molecular oxygen, or photo-oxidation. They are therefore prone to various structural alterations as a consequence of the disruption of the cell and chloroplast during grinding, cooking, processing, and/or digestion of the foodstuff (*4*, *6*, *7*).

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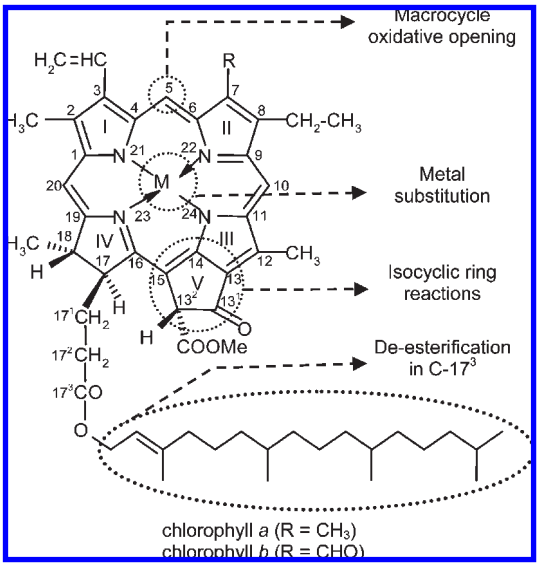

Figure 1. Main structural transformations of the chlorophyll molecule.

The broad multiplicity of chlorophyll derivatives (**Figure 1**) originated both before and after ingestion of the green vegetable has greatly hampered the study of their functionality. For this reason sodium copper chlorophyllin (SCC), a metastable chlorophyll derivative, has been employed generically in such studies. SCC is a commercial preparation consisting of a synthetic mixture of water-soluble cuprosodium salts, obtained by saponification of the chlorophyll and subsequent addition of Cu. The final product is not a pure compound but a mixture of chlorin-type compounds, in which Cu-chlorin e_4 and Cu-chlorin e_6 are the major ones (*8*). Although this preparation offers the advantages of hydrosolubility, stability in solution, and ready acquisition on the market, its varied composition, depending on the starting material and processing system, makes exceedingly difficult the attribution of a specific biological function to a particular component.

Depending on the structural changes of the native molecule of chlorophyll, there will be modifications in the solubility, molecular properties, and/or reactivity of the original compound and, as a consequence, in its primary functional properties (*7*). Thus, inhibition of lipid hydroperoxide formation has been demonstrated for some phorphyrin structures (*9*), and the importance of the formyl group at C-7 or the ion metal in the porphyrin ring for the antioxidant capacity has been shown (*10*, *11*). The protective effect against direct-acting mutagens also seems to be related to structural differences, the dephytylated chlorophyll derivatives being less efficient (*12*). The recently discovered capacity of the tetrapyrroles to induce activity of the phase II detoxifying enzymes, protecting cells against oxidants and electrophiles, also seems to be modulated by structural differences (*13*).

In this regard, the association between a high risk of colon cancer and a diet rich in processed red meat and low in green vegetables (*14*) has led to the study, in animal models, of the interaction between the hemo group, the pro-oxidant porphyrin pigment of red meat, and the vegetable diet. The results have demonstrated that the intake of green vegetables may reduce the risk of colon cancer because the chlorophylls inhibit the cytotoxic effects of the hemo group in the colon mucosa. However, this anti-cytotoxic activity is specific to natural chlorophylls, and it could not be demonstrated with the commercial preparation, SCC (*15*). The results obtained to date demonstrate the need for a broad study to suggest dietary healthy habits or chlorophyll-type supplements aimed at the chemoprevention of degenerative diseases.

The aim of this work was to assess the bioaccessibility of chlorophyll compounds from an oily food matrix. With this object, the fatty food was subjected to an in vitro digestion standard process developed by Garret et al. (*16*). This protocol reproduces the physiological conditions of gastric and intestinal phases and has been widely used to assess the digestive stability and bioaccesibility of different phytochemicals from foods (*17*, *18*).

Dietary fats must be digested in the lumen of the small intestine before the body can assimilate them. In this digestion process, their major components (triacylglycerols) are partially hydrolyzed into monoacylglycerols and fatty acids by the action of digestive enzymes. The bile salts, by their amphiphatic character, play a key role in the formation of molecular aggregates with these hydrolyzed products and other lipophilic components such as cholesterol and carotenoids. The resulting colloid dispersion allows these compounds to be absorbed across the intestinal epithelial cell (enterocyte) membranes (*19*). The general mechanism of chlorophyll compound absorption can be expected to be similar to that of the above lipophilic xenobiotics, via their molecular aggregation in transport micelles (*1*). The work planning was focused on studying the digestive stability, the capacity of dispersion in the colloid system, and the mechanism of absorption by the intestinal epithelial cells of the native chlorophylls (*a* and *b*) and of the main chlorophyll derivatives generated during the processing and/or digestion of green vegetables: pheophytin, pheophorbide, pyropheophytin, and pyropheophorbide.

MATERIALS AND METHODS

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

Chemicals and Standards. 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 (FBS), penicillin—streptomycin—glutamine, nonessential amino acids, Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were purchased from Gibco (Invitrogen Ltd., Paisley, U.K.). Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands), HPLC reagent grade solvents were purchased from Teknokroma (Barcelona, Spain), and analytical grade solvents were supplied by Panreac (Barcelona, Spain). For the preparation, isolation, and purification of chlorophyll pigments, analytical grade reagents were used (Panreac). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Bedford, MA).

Standards of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and β-carotene were supplied by Sigma-Aldrich Co. Standards of pheophytin *a* (phy *a*) and pyropheophytin *a* (pyrophy *a*) were provided by Wako Chemicals Gmbh (Neuss, Germany), and standards of pheophorbide *a* (pho *a*) and pyropheophorbide *a* (pyropho *a*) were purchased from Frontier Scientific Europe Ltd. (Carnforth, Lancashire, U.K.). The C-13 epimer of chl (*a* or *b*) was prepared by treatment with chloroform according to the method of Watanabe et al. (*20*). 13^2-OH-chl (*a* or *b*) was obtained by selenium dioxide oxidation of chl at reflux heating for 4 h in pyridine solution under argon (*21*). 15^1-OH-lactone-chl (*a* or *b*) was obtained from chl by alkaline oxidation in aqueous media according to the method of Mínguez-Mosquera and Gandul-Rojas (*22*).

Preparation of Chlorophyll Pigments for Digestion Assays. Chls *a* and *b* were extracted from fresh spinach leaves by using acetone and transfer to diethyl ether. Subsequently, they were separated and isolated by TLC on silica gel GF_{254} using as developing mixture petroleum ether 65−95 °C/acetone/diethylamine (10:4:1). Bands for chl *a* and chl *b* were scraped from the plate at R_f 0.51 and 0.44, respectively, and eluted with acetone (*7*). Phys *a* and *b* were prepared from pure chl *a* or chl *b* diethyl ether solutions, respectively, by acidification with a few drops of HCl 13% (v/v) (*23*). Pho *a* was formed from phy *a* by enzymatic de-esterification: a

protein precipitate of *Ailanthus altissima* (Mill.) leaves was extracted with 5 mM sodium phosphate buffer (pH 7.0), containing 50 mM KCl and 0.24% (w/v) Triton X-100, and incubated with 100 mM Tris-HCl buffer (pH 8.5) containing 0.24% (w/v) Triton X-100 and the substrate dissolved in acetone in a 5:5:1 ratio (*24*). Pyrophy *a* and pyropho *a* were prepared from pure phy *a* or pho *a* pyridine solutions, respectively, by reflux heating during 8 h at 115 °C (*25*).

All chlorophyll pigments were purified by semipreparative HPLC, and they were >97% pure as judged by HPLC. Stock solutions of chl *a*, chl *b*, phy *a*, phy *b*, pyrophy *a*, pho *a*, and pyropho *a* were prepared in acetone (~2 mM).

In Vitro Digestion. Different test meals were prepared by adding a chlorophyll pigment to commercial sunflower oil. Three hundred and forty microliters from the stock solution (0.68 μmol) was evaporated to dryness under nitrogen and subsequently dissolved in sunflower oil (300 μL) for each independent digestion assay (eight per pigment). The digestive stability was assessed by using the in vitro digestion procedure developed by Garret et al. (*16*), which reproduces physiological conditions of the gastric phase, including homogenization of test meal with 10 mL of saline solution (140 mM NaCl, 5 mM KCl) under vigorous stirring, acidification to pH 2.0 with 0.1 M HCl, and addition of porcine pepsin (2.4 mg/mL). The mixture was incubated at 37 °C for 1 h in a thermostated water bath with magnetic stirring at 500 rpm (gastric digestion).The pH of gastric digesta was then increased to 6 with 0.9 M $NaHCO_3$, and bile extract and pancreatin in 0.1 M $NaHCO_3$ were added to provide final concentrations of 2.4 and 0.4 mg/mL of digesta, respectively, in a final reaction volume of 16.55 mL. The pH was elevated to 7.0 with 2 M NaOH, and then the small intestinal phase was initiated by incubation in a thermostated water bath at 37 °C for 2 h with magnetic stirring at 500 rpm. The final concentration of each pigment was ~41.1 μM. Upon completion of the phases, aliquots (2 × 1 mL) of the digesta were transferred to 15 mL tubes, blanketed with nitrogen, and stored at −20 °C until analysis for determination pigment stability during simulated digestion.

The aqueous colloid system containing the molecular aggregates resulting from the digestion was isolated by ultracentrifugation from solid particles and residual oil not hydrolyzed, according to the method developed by Hernell et al. (*26*). Those authors showed that in this aqueous emulsion coexist micelles (size ≤ 40 Å) and unilamellar vesicles (size = 200−600 Å). Samples (14 mL) of digesta were ultracentrifuged at 167000*g* at 4 °C for 40 min. The intermediate aqueous dispersion was carefully collected with a Pasteur pipet (230 mm length), avoiding contamination with the floating oil droplets and the pellet. In the same way as Garret et al. (*16*), the collected aqueous phases were finally filtrated (0.22 μm pore size) to remove contaminating aggregates with size > 2000 Å and operationally were called the "aqueous-micellar fraction" (AMF). Each AMF was refrigerated at 4 °C for cellular uptake experiment (see below). Aliquots (2 × 1.5 mL) were separated, blanketed with nitrogen, and stored at −20 °C until pigment analysis to assess micellarization of chlorophyll pigments. The associated variability to this in vitro digestion procedure, which stems from the different physical and biochemical stages in it, was resolved by increasing to eight the number of independent digestion assays per pigment. Thus, each experiment can be differentiated and appropriate conclusions can be obtained.

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 a 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 confluence. Prior to the chlorophyll absorption experiments, the last two medium changes were carried out using serum-free medium.

Uptake of Chlorophyll Pigments by Human Intestinal Epithelial Cells. Assays of absorption by Caco-2 cell monolayers were performed according to the method of Garret et al. (*16*). The potential cytotoxicity of the AMFs 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 lipid molecular aggregates did not adversely affect cellular integrity.

The AMFs obtained from the in vitro digestion process were appropriately diluted (if necessary) with saline solution to obtain the same concentration (0.2 μM) for all test foods and facilitate direct comparison of results. The diluted AMFs were mixed with basal DMEM in a 1:3 (v/v) ratio (test medium), and 25 mL was added to each monolayer; cultures were incubated at 37 °C for 5 h. Eventually, the medium was removed, and the monolayers were washed twice 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 2 × 2 mL of ice-cold phosphate-buffered solution 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 digesta, AMF, and Caco-2 cells, with the mixture acetone—diethyl ether, as is described in a previous work (*5*). HPLC analysis of chlorophyll pigments was performed according to the method described by Mínguez-Mosquera et al. (*27*), 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 pigments were identified by cochromatography with the corresponding standard and from their spectral characteristics described in detail in previous papers (*22*, *27*, *28*). The online UV—vis spectra were recorded from 350 to 800 nm with the photodiode array detector. Detection of pigments was performed at 410, 430, 450, and 666 nm and quantified from the corresponding calibrate curves.

Calculations and Statistical Analysis of Data. HPLC analysis of each pigment solution used for digestion was performed in triplicate. The profiles of chlorophyll pigments in test meals after in vitro digestion process were determined from eight independent digestions. Digestas and AMFs were extracted in duplicate for HPLC pigment analysis. Percentage of pigment recovery in digesta (D) was calculated as nanomoles in D × 100/nanomoles in starting material. Percentage of pigment solubilization in AMF (operationally designed "micellarization") was calculated as nanomoles in AMF × 100/nanomoles in D. This gives information of the proportions of those pigments present in digesta that are ready for the enterocyte absorption.

For cellular studies, the diluted AMF corresponding to each test meal was added to six replicate flasks with 14 days postconfluent Caco-2 cell monolayers. The uptake of chlorophyll derivatives by Caco-2 cells was expressed as rate of absorption per milligram of protein (pmol × min^{-1} × mg^{-1} of protein). For comparative assays the percentage of absorption and apparent permeability coefficient (P_{app}) were obtained. The first was calculated as nanomoles of pigment added to the cell monolayer × 100/nanomoles accumulated by the cell monolayer, and the second was calculated in a similar way to Artursson et al. (*29*) as

$$P_{app} = (1/C_0) \times \Delta Q/\Delta t$$

where C_0 is the initial concentration in the donor chamber (μM) and $\Delta Q/\Delta t$ is the absorption rate per protein unit.

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), a Büchi rotavapor, model R 110 (Laboratoriums-technik AG), an MLA-80 rotor used in a Beckman Coulter Optima MAX ultracentrifuge (Palo Alto, CA), and an HP 1100 Hewlett-Packard (Palo Alto, CA) liquid chromatograph fitted with an HP 1100 automatic injector and an HP 1100 photodiode array detector.

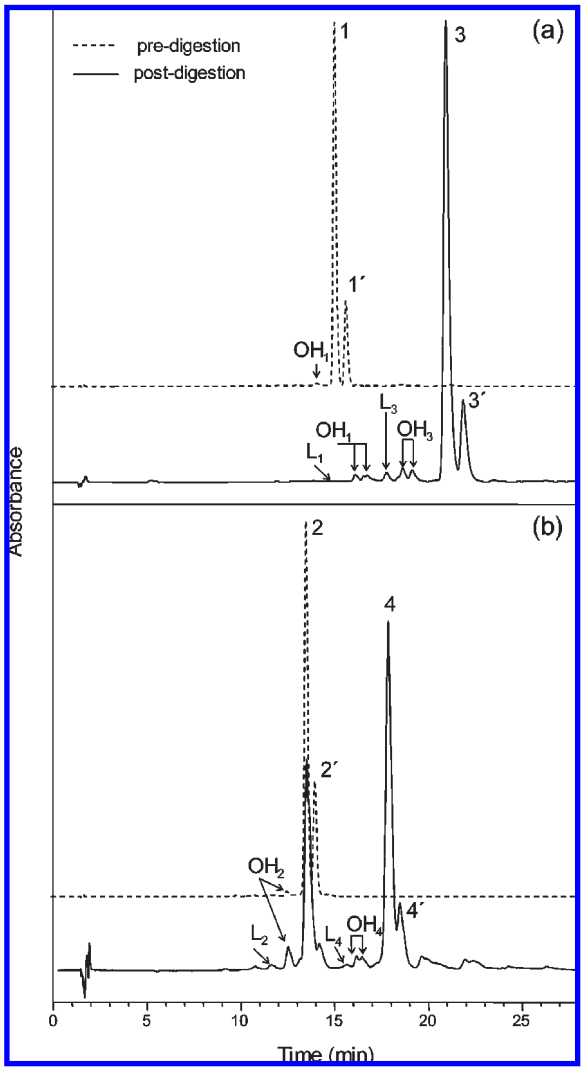

Figure 2. HPLC chromatograms of pigment extracts obtained from test meal before (predigestion) and after (postdigestion) simulated in vitro digestion. Test meal comprised standard pigment dissolved in an oily matrix. Standard pigments were (**a**) chlorophyll *a* and (**b**) chlorophyll *b*. Peaks: 1, chlorophyll *a*; 1′, chlorophyll *a*′, L_1, 15^1-OH-lactone chlorophyll *a*; OH_1, 13^2-OH-chlorophyll *a*; 2, chlorophyll *b*; 2′, chlorophyll *b*′; L_2, 15^1-OH-lactone chlorophyll *b*; OH_2, 13^2-OH-chlorophyll *b*; 3, pheophytin *a*; 3′, pheophytin *a*′; L_3, 15^1-OH-lactone pheophytin *a*; OH_3, 13^2-OH- pheophytin *a*; 4, pheophytin *b*; 4′, pheophytin *b*′; L_4, 15^1-OH-lactone pheophytin *b*; OH_4, 13^2-OH-pheophytin *b*. Detection was at 430 nm for chlorophyll *a* and pheophytin *b*, at 410 nm for pheophytin *a*, and at 450 nm for chlorophyll *b*.

RESULTS AND DISCUSSION

Digestive Stability. A comparative assay was designed to study the digestive stability of chl *a* and chl *b* in an oily food matrix. The test meal comprised standard pigment dissolved in high-oleic sunflower seed oil. This type of oil was chosen as being a good source of lipids not containing chlorophyll pigments that could interfere in the experiment and for its relative resistance to autoxidation reactions. The lipids are necessary to form the micelles and vesicles for transporting hydrophobic components, after being hydrolyzed during the digestion process and together with bile salts (*19*).

Figure 2 shows representative HPLC chromatograms of the extracts from the test meal before (predigestion) and after (postdigestion) in vitro digestion, for chl *a* (**Figure 2a**) and chl *b* (**Figure 2b**). The results of the quantitative analysis, expressed as nanomoles of pigment per milliliter of digesta, are gathered in

Table 1. Changes in Pigment Profile and Content (Nanomoles per Milliliter) after Simulated in Vitro Digestion of Test Meal Comprising Chlorophyll *a* or Chlorophyll *b* in an Oily Matrix

	chlorophyll *a*		chlorophyll *b*	
chlorophyll pigment[a]	predigestion[b]	postdigestion[c]	predigestion[b]	postdigestion[c]
chl	50.55 ± 0.01	0.08 ± 0.01	35.80 ± 0.20	5.41 ± 0.40
13^2-OH-chl	0.70 ± 0.07	0.64 ± 0.06	0.86 ± 0.04	1.61 ± 0.43
15^1-OH-lactone-chl		0.15 ± 0.02		0.06 ± 0.06
phy		23.35 ± 0.54		10.32 ± 0.56
13^2-OH-phy		1.03 ± 0.04		0.46 ± 0.04
15^1-OH-lactone-phy		0.99 ± 0.05		0.25 ± 0.02
total chls	51.25 ± 0.05	26.24 ± 0.49	36.66 ± 1.24	18.11 ± 0.93

[a] Abbreviations: chl, chlorophyll; phy, pheophytin. [b] Data represent the mean value ± SE ($n = 3$). [c] Data represent the mean value ± SE for eight independent digestions, each analyzed in duplicate.

Table 1. The digestive stabilities of the native chlorophylls to the gastric phase acidity (pH 2.0) were different for chl *a* and chl *b*. Thus, in the case of digestion of chl *a*, around 97% of the chlorophyll pigments were Mg-free derivatives (peaks 3, 3′, L_3, and OH_3 in **Figure 2a**), whereas for chl *b* there was a high percentage of chlorophyll derivatives with Mg (39%) (peaks 2, 2′, OH_2, and L_2 in **Figure 2b**). The highest stability of chl *b* to acidic conditions has been previously established in studies on thermal processes or fermentation of green vegetables. It is known that the formyl group at C-7 of chl *b* interferes sterically in the pheophytinization reaction and that the rate of this reaction is always higher for chl *a*.

By the 1930s, Mg-free chlorophyll derivatives had already been identified in human feces (*1*) and, more recently, Ferruzzi et al. (*4*) have demonstrated that when spinaches are submitted to an in vitro digestion system (37 °C, pH 2.0, 1 h), the chlorophylls are completely transformed into pheophytins. That study did not rule out that chlorophylls could remain intact after a process of digestion under specific physiological and/or physicochemical conditions able to maintain a higher pH, of 4−6 units. In a recent study, carried out with various forms of processed peas (*5*), we have also found that during the in vitro digestion of the peas, the pheophytinization reaction is almost complete, 13^2-OH chls *a* and *b* being the only Mg-containing chlorophyll derivatives formed in some of the test foods, and at very small proportions. In the present study, it could be possible that because the chlorophyll pigments were directly dissolved in oil for preparing the test food, a protective effect on the pigments was promoted.

During the gastric phase dephytylated chlorophyll derivatives were not detected, although it has been reported in a model system (*23*) that strongly acidic conditions may provoke de-esterification of pheophytins to give pheophorbides. It has been demonstrated (*4*, *5*) that this type of reaction does not occur in the phase of gastric digestion or in the small intestine, associating the detection of pheophorbides in human feces with the action of the large intestine flora.

Allomerization reactions involving C-13^2 oxidation by triplet molecular oxygen were also found during the in vitro digestion process. These allomerization reactions were not significantly different ($p > 0.05$) for chl *a* and chl *b*, the allomerized derivatives being increased from 1.37 ± 0.16 to 10.71 ± 0.61% for chl *a* and from 2.35 ± 0.24 to 13.14 ± 0.68% for chl *b* (percentage with respect to the total chlorophyll compounds). The allomerized derivatives identified in both cases were 13^2-OH-chlorophylls (peaks OH_1 and OH_2), 15^1-lactone-chlorophylls (peaks L_1 and L_2), 13^2-OH-pheophytins (peaks OH_3 and OH_4), and 15^1-lactone-pheophytins (peaks L_3 and L_4). This oxidation reaction can take place both enzymatically and chemically (*30*). Recently, in

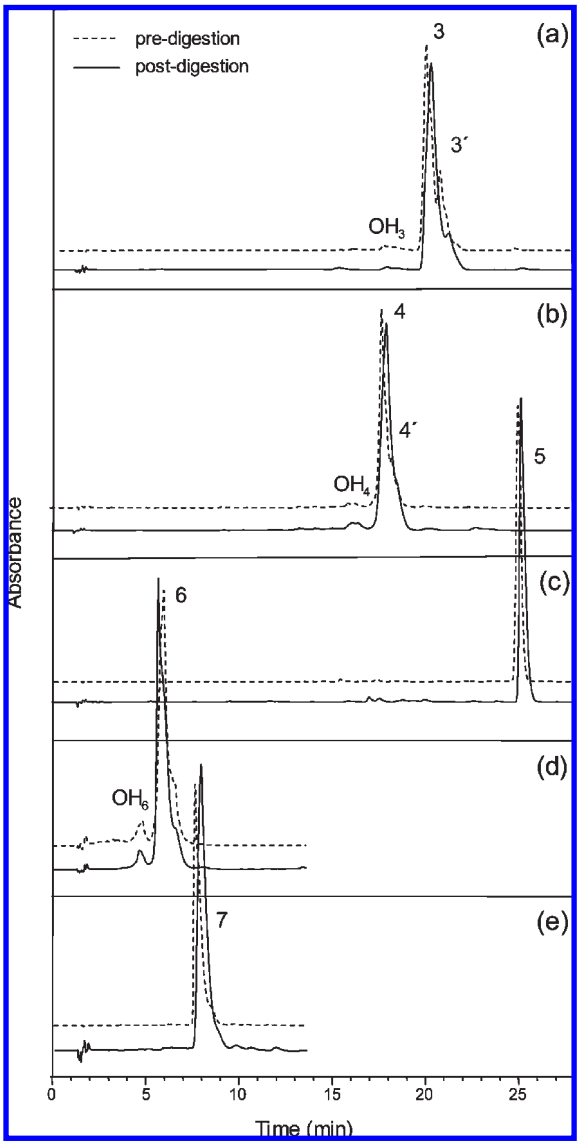

Figure 3. HPLC chromatograms of pigment extracts obtained from test meal before (predigestion) and after (postdigestion) simulated in vitro digestion. Test meal comprised standard pigment dissolved in an oily matrix. Standard pigments were (**a**) pheophytin *a*, (**b**) pheophytin *b*, (**c**) pyropheophytin *a*, (**d**) pheophorbide *a*, and (**e**) pyropheophorbide *a*. See **Figure 2** for peaks 3, 3′, OH_3, 4, 4′, and OH_4. Peaks: 5, pyropheophytin *a*; 6, pheophorbide *a*; OH_6, 13^2-OH-pheophorbide *a*; 7, pyropheophorbide *a*. Detection was at 410 nm for pheophytin *a*, pyropheophytin *a*, pheophorbide *a*, and pyropheophorbide *a* and at 430 nm for pheophytin *b*.

studies on bioaccessibility of chlorophylls from fresh and processed peas (*5*), we have detected allomerization reactions only in the digestion of fresh pea, and we have related this transformation with a peroxidase enzymatic activity. However, in the present work, the in vitro digestion had been carried out with pure pigments, and the only enzymes that were present in the medium were those of digestion, so the origin of the allomerization reactions should be purely chemical.

The other studied pigments were the Mg-free chlorophyll derivatives phy *a*, phy *b*, pyrophy *a*, pho *a*, and pyropho *a*. **Figure 3** shows the representative HPLC chromatograms of each in vitro digestion process. In contrast to chls *a* and *b*, the chromatographic profiles of the Mg-free derivatives dissolved in the lipid matrix remained virtually unaltered after gastric and intestinal digestion, and only a small increase in the allomerized

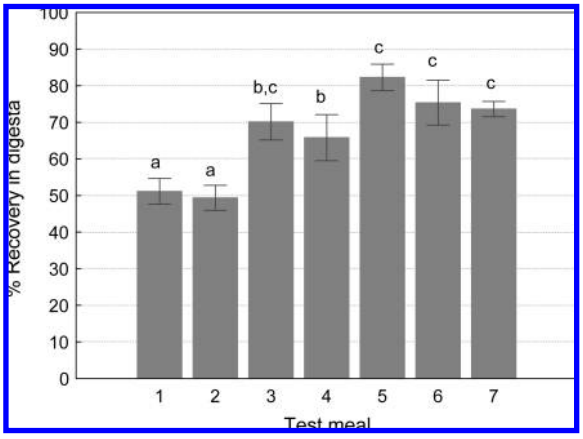

Figure 4. Percentage of total chlorophyll pigments recovered after simulated in vitro digestion of test meals comprising standard pigment dissolved in an oily matrix. Standard pigments: 1, chlorophyll *a*; 2, chlorophyll *b*; 3, pheophytin *a*; 4, pheophytin *b*; 5, pyropheophytin *a*; 6, pheophorbide *a*; 7, pyropheophorbide *a*. Values are means ± SE for eight independent digestions, each analyzed in duplicate. Different letters above the error bars indicate significant differences between test meals in the recovery values ($p < 0.05$).

pigment percentage (around 0.5−4%) was observed. Although the Mg-free chlorophyll derivatives can also undergo allomerization, the reaction rate is slower than for the native chlorophylls (*31*).

Finally, it was checked whether the oxidation reactions during the digestion process also involved the cleavage of the porphyrin ring, giving rise to colorless products. With that purpose, the percentage of total chlorophyll pigments recovered in the digesta, with respect to the initial content in the test food, was calculated (**Figure 4**). In overall terms, retentions of chl *a* and chl *b* after digestion were not significantly different ($p > 0.05$), showing in both cases pigment destruction of around 50%. With regard to the rest of pigments, the retentions after digestion were of the same order for all of them (phy *a*, 70.2 ± 5.0%; phy *b*, 65.8 ± 6.3%; pyrophy *a*, 82.3 ± 3.6%; pho *a*, 75.4 ± 6.1%; and pyropho *a*, 73.6 ± 2.1%) and significantly higher ($p < 0.05$) than that of chlorophylls.

It appears from these results that, in general, the native chlorophylls show greater instability to the digestive process than the Mg-free chlorophyll derivatives, because in addition to pheophytinization reactions, the allomerization and the oxidation to uncolored compounds were found to greater extent for the former.

Dispersion in the AMF. The efficiency of the compound transfer from the digesta to the AMF was expressed as a percentage of "micellarization" (**Figure 5**). As mentioned in the previous section, after the in vitro digestion process, chl *a* was almost entirely (97%) transformed to Mg-free derivatives, whereas chl *b* was around 61%. As a result, it was not possible to obtain the micellarization percentage of native chlorophylls, but it was the micellarization percentage of the mixture (chl and phy). The efficiency of the transfer to the AMF was not significantly ($p > 0.05$) different when the test meal was chl *a* (25.5 ± 4.3%) or phy *a* (32.6 ± 5.9%). With regard to the experiment with the chlorophyll compounds of the series *b*, there were also no significant ($p > 0.05$) differences between the micellarization of the mixture chl *b* and phy *b* (10.2 ± 1.9%) and the corresponding when phy *b* was the starting material (14.36 ± 4.24%). On the contrary, the micellarization of phy *a* was significantly higher ($p < 0.05$) than that of phy *b*. This result was not in agreement with data obtained previously for us for the study carried out on bioaccessibility of

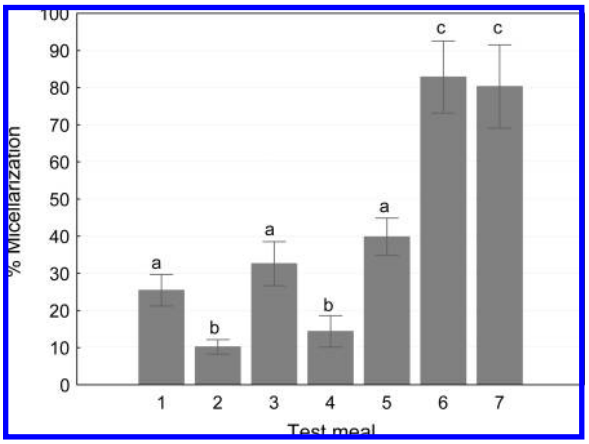

Figure 5. Percentage of transference of total chlorophyll pigments from the digesta to the aqueous micellar fraction during simulated in vitro digestion of test meals comprising standard pigment dissolved in an oily matrix. See **Figure 4** for standard pigment numbers. Data represent the mean value ± SE for eight independent digestions, each analyzed in duplicate. Different letters above the error bars indicate significant differences between test meals in the micellarization of total chlorophyll derivatives ($p < 0.05$).

chlorophylls from peas, in which the test food included the whole of the pea pigments (*5*). It is known for some carotenoids that their transfer to micelles may be affected by the presence of other carotenoids (*32*, *33*), and we thought that a similar phenomenon could happen for chlorophyll pigments. With the aim of checking this hypothesis, an in vitro digestion experiment with the phy *a* and phy *b* mixture was conducted. The results showed no significant differences between the solubilization in the AMF for both compounds (44.55 ± 4.28 and 45.45 ± 1.99% for phy *a* and phy *b*, respectively), because a great increase, around 31%, in the micellarization degree of phy *b* was obtained. Although the transfer of phy *a* to the AMF (44.55 ± 4.28%) was also apparently higher than when the in vitro digestion process was made with the isolated pigment (32.6 ± 5.9%), that increase was not significant ($p > 0.05$). This test revealed that the presence of phy *a* had a positive effect in the micellarization of phy *b* and could explain the above result obtained with the pea study.

The absence of a carbomethoxy group at C-13[2] of the chlorophyll molecule did not lead to significant differences ($p > 0.05$) in the micellarization degree, with regard to the precursor compound, resulting for pyrophy *a* at 39.9 ± 5.0%. However, the loss of phytol led to both pho *a* and pyropho *a* showing micellarization levels (84.1 ± 8.3 and 80.3 ± 11.2%, respectively), significantly higher ($p < 0.01$) than those for phy *a* and pyrophy *a*.

Ferruzzi et al. (*4*) found in fresh spinach puree a significantly lower micellarization degree for the mixture of chl *b* and phy *b* than for the mixture of chl *a* and phy *a*; the difference was less pronounced after the heat treatment of the puree and became insignificant in puree treated with Zn(II) salts. In the same study, other pigments, such as lutein and β-carotene, for which the difference in liposolubility is more marked than that between chl *a* and chl *b*, did not show significant differences in the degree of micellarization in the fresh puree, whereas in Zn(II)-treated puree, the micellar solubilization decreased very significantly ($p < 0.01$) for β-carotene but not for lutein. In summary, although it seemed logical to think that there was a relationship between the molecule polarity and its ability to dispersion in the AMF, all of these results demonstrate that this one is not the only factor determining the extent of its micellarization; other factors involving both the type of food matrix and the processing system, which may involve degree of transformation in the digestive process, have to be taken into account when the potential bioaccessibility of the various chlorophyll derivatives is evaluated.

In the case of pho *a* and pyropho *a*, the pronounced increase in their hydrosolubility, which is associated with phytol de-esterification, is correlated with a very significant increase in their potential bioaccessibility. Hence, a mild heat treatment of the vegetable matrix, at temperatures not exceeding 80 °C, promoting the activation of the enzyme chlorophyllase, could greatly improve the bioaccessibility of the chlorophyll compounds.

Uptake by Human Intestinal Epithelial Cells. For the uptake assays of chlorophyll derivatives by Caco-2 cells monolayers the total concentration of chlorophyll pigments in the cell medium could vary from 0.2 to 1.5 μM, depending on the micellarization degree of each tested compound. These concentrations were in the range used for other lipophilic pigments, such as for synthetic micelles of β-carotene (0.12−22 μM) (*34*) or lutein (1.5−15 μM) (*35*). In the present study, and with the aim of performing comparative assays of absorption, the AMFs obtained from the in vitro digestion process were diluted with saline solution to obtain a concentration of 0.2 μM. After mixing with basal DMEM (1:3 v/v), the pigment concentration in the cell medium was 0.05 μM.

Figure 6 shows the HPLC chromatograms of pigment extracts obtained from the cell cultures, at 410 and 666 nm for the chlorophyll pigments of the series *a* and at 450 and 654 nm for chl *b*. The analysis were performed after 5 h of incubation of the Caco-2 cells with diluted AMFs from the digestion of chl *a* (**Figure 6a**), chl *b* (**Figure 6b**), pyrophy *a* (**Figure 6c**), pho *a* (**Figure 6d**), and pyropho *a* (**Figure 6e**). As shown in **Figure 6a**, **b**, all of the pigments derived from the digestion of the chlorophylls, including pheophytins, OH-pheophytins, and lactone-pheophytins, were absorbed by the epithelial cells. Moreover, in contrast to the findings of earlier studies, there was also a cellular uptake of Mg-containing chlorophyll derivatives, in the products from chl *b* digestion. Quantitatively, the uptake percentages ranged from 3 to 7%; no significant differences were found between the uptake of compounds from the digestion of chl *a* and that from the digestion of chl *b* and pyrophy *a*. A moderately efficient uptake (4−10%) of liposoluble chlorophyll derivatives from spinach puree (*4*) and processed peas (*5*) has also been reported. With regard to the dephytylated compounds, the absorption percentages were significantly higher, being 9% for pyropho *a* and 26% for pho *a*.

On the other hand, the chromatograms at 410 and 450 nm showed the detection of a non-chlorophyll compound (peak marked with an asterisk), which was identified as β-carotene. During the cell growth and 10 days after confluence, the cells were grown in medium with fetal bovine serum. An analysis of pigments made in this culture medium resulted in 0.03 μM β-carotene, explaining its detection in the absorption experiments. To overcome this interference, the quantification was performed at 666 and 654 nm.

Rate of Absorption and Transport. To reveal possible differences in the rate and mechanism of transport for the chlorophyll compounds, comparative assays of phy *a*, pyrophy *a*, pho *a*, and pyropho *a* uptake by Caco-2 cells at various initial concentration levels in the cell culture medium were performed (**Figure 7**). The results showed marked contrasts, both in the maximum uptake percentage and in the shape of the cellular uptake increase for each pigment, depending on the initial concentration.

In general, the rate of pigment accumulation by the cell culture increased with the initial concentration in the AMF following a passive transport down the concentration gradient. In the case of phy *a* and pyrophy *a* (**Figure 7a**), the increase was linear

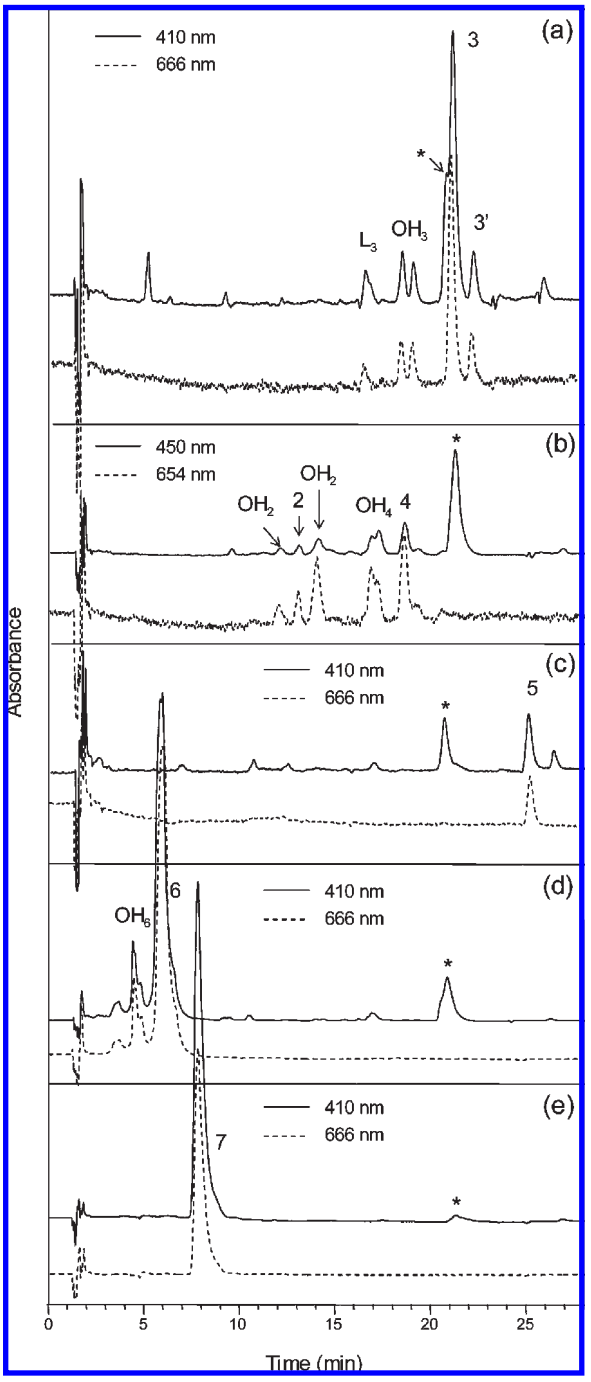

Figure 6. HPLC chromatograms of pigment extracts obtained from the Caco-2 cell cultures after 5 h of incubation with aqueous micellar fractions from the simulated in vitro digestion of test meals comprising standard pigment dissolved in an oily matrix. Standard pigments: (**a**) chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) pyropheophytin *a*; (**d**) pheophorbide *a*; (**e**) pyropheophorbide *a*. Peaks are as in **Figures 2** and **3**. Peak with an asterisk: β-carotene.

throughout the assayed range, demonstrating a simple diffusion mechanism in the passive transport for both compounds. In contrast, the cellular uptakes of pho *a* and pyropho *a* (**Figure 7b**) were different. In these cases, the cellular uptake rate increased to 100 and 30 pmol $\times$ min^{-1} $\times$ mg^{-1} of protein for pho *a* and pyropho *a*, respectively, at the lower concentrations in the cell media and then remained without significant differences ($p > 0.05$) up to around 0.5 μM. This indicated a decrease in the percentage of pigment uptake, which suggested that in that concentration interval there was saturation in the passive transport and thus a

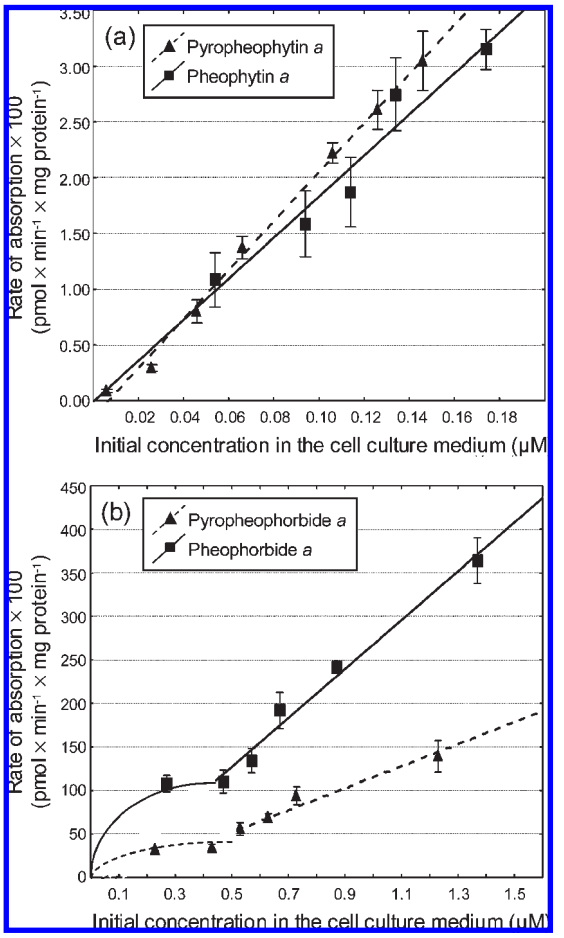

Figure 7. Rate of pigment accumulation by the Caco-2 cell cultures at various initial concentration levels in the cell culture medium: (**a**) pheophytin *a* and pyropheophytin *a*; (**b**) pheophorbide *a* and pyropheophorbide *a*.

facilitated diffusion mechanism. For concentrations >0.5 μM, there was a constant increase in the uptake rate, demonstrating a simple diffusion mechanism in the passive transport.

It has been reported that for certain drugs, transport is not by a single mechanism but can be partially facilitated by a carrier and also follow a simple difussion pathway (*29*). As facilitated transport is saturable, the contribution of simple diffusion mechanism in the passive transport can increase with the increase in concentration. Our experiments showed evidence that the mechanism of pho *a* and pyropho *a* transport by the epithelial cells was mixed. At low concentrations, the uptake rate was higher than the one that would be obtained by an exclusively simple diffusion mechanism, as there was a partial contribution from facilitated diffusion transport by membrane carriers. At higher concentrations, once the possible carrier had become saturated, the transport mechanism was exclusively by simple diffusion.

Using flow cytometry, Jonker et al. (*36*) demonstrated differences in the relative uptake of pho *a* by the ovarian cancer cell line (IGROV1) and its derived line T8, which is overexpressed in BCRP, a protein involved in the control/elimination of cancerigenous drugs. They showed that bcrp1 efficiently reduces the bioavailability of dietary pho, impeding its uptake by the intestine and possibly mediating in its elimination via the liver and kidney. They also demonstrated in mice mutant on this carrier (bcrp1$-/-$) that without this efficient natural barrier, the greater uptake of pho *a* resulting from fluctuations of pho *a* in the diet can cause phototoxicity. Robey et al. (*37*) found that pho *a* is a specific substrate of the human carrier ABCG2, and they have

developed a flow cytometry assay to determine specifically the expression and function of ABCG2 using pho *a*.

It is important to emphasize that the quantitative valuation of the uptake rate carried out in this work has enabled very significant differences to be found between the various chlorophyll derivatives, for the maximum percentage of uptake reached (the apparent permeability coefficient, P_{app}). Whereas phy *a* and pyrophy *a* showed similar P_{app} (18.7 ± 1.4 and 18.3 ± 1.0 $min^{-1} \times mg^{-1}$ of protein, respectively), the obtained value for pyropho *a* was almost 5 times higher, and the value for pho *a* was significantly higher by 2 orders of magnitude (292 ± 15 $min^{-1} \times mg^{-1}$ of protein).

It was therefore demonstrated that the transport of these compounds by the intestinal epithelial cells was not exclusively by simple diffusion as might be assumed in principle—evidence was found for the first time of the participation of a facilitated diffusion transport for the dephytylated derivatives, pho *a* and pyropho *a*.

Lohrey et al. (*38*) found that albino rats fed lucerne−protein concentrate develop photosensitization, suggesting that it may be due to pheophorbides or other related pigments, because they found substantial amounts of pho *a* in the blood plasma and liver of the rats. The authors suggested the hypothesis that these smaller molecules can be more readily absorbed through the intestinal wall. However, photosensitization was not observed in rats fed ryegrass−protein concentrate, this difference being likely caused by the difference in chlorophyllase activity of the two plant species. In relation to this conclusion, Holden (*39*) found that the dephytylated chlorophyll derivative content of the leaf protein preparations is correlated with the chlorophyllase activity of the leaves and with the thermal conditions of the processing and storage. To avoid the formation of dephytylated pigments, the denaturalization of the protein at temperatures close to 100 °C is suggested.

Recently, high contents of pho *a* and pyropho *a* have been found in dried laver product (called nori) implicated in food poisoning, in which some persons developed allergic reaction to sunlight (*40*). Because the light absorption properties of phy *a* and pho *a* are identical, and according to the results found in this work, we suggest that the phototoxicity traditionally associated with pho *a* and pyropho *a* might not be due to the type of molecular structure but to the greatest bioaccessibility, leading to a higher intestinal absorption of these compounds. Likewise, the demonstration of a facilitated transportation for the dephytylated chlorophyll derivatives allow interpretation of the fact that only certain individuals develop such phototoxicity: they may be deficient in bcrp, a protein that regulates pho transport.

In conclusion, structural differences in the chlorophyll pigment molecule significantly affected its transfer from the food matrix to the intestinal epithelial cells during digestion. Mainly, the de-esterification of the alcohol phytol produced a very significant increase both in the efficiency of the transfer to the aqueous micellar fraction and in the uptake rate by the Caco-2 cells. For phy *a*, an approximate calculation showed that the bioaccessibility of the chlorophyll compound will increase 65-fold if it is in the form of pho *a*. Moreover, differences in the transport route were also found. Whereas phytylated chlorophyll derivatives showed absorption by simple passive diffusion, the dephytylated ones showed facilitated diffusion transport in the lower range of concentrations tested.

ABBREVIATIONS USED

chl, chlorophyll; phy, pheophytin; pyrophy, pyropheophytin; pho, pheophorbide; pyropho, pyropheophorbide; SCC, sodium copper chlorophyllin; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; BHT, butylated hydroxytoluene; D, digesta; AMF, aqueous micellar fraction, Papp, apparent permeability coefficient.

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