FIRST-PASS METABOLISM OF CHLOROPHYLLS IN MICE

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List of abbreviations:

ABC: ATP-binding cassette; ADME: absorption, distribution, metabolism and excretion; AMF: aqueous micellar fraction; APCI: atmospheric pressure chemical ionization; BHT: butylated
hydroxytoluene; BLT1: blocks lipid transport 1; EIC: extracted ion chromatogram; hrTOF: high resolution TOF; PDT: photodynamic therapy; Qq: quadrupole-quadrupole; SR-BI: scavenger receptor B type I; UHR: ultra-high resolution

Keywords: chlorophylls; absorption; liver; pheophorbide; SR-BI.
Abstract

Scope: The dietary intake of chlorophylls is estimated to be approximately 50 mg/day. However, their first pass metabolism and systemic assimilation is not well characterized.

Methods and results

A group of 30 mice were fed a diet rich in chlorophylls, while 10 mice received a standard diet without chlorophylls (control group). Liver extracts were analyzed every 15 days by HPLC-ESI(+)/APCI(+)-hrTOF- MS/MS to measure the accretion of specific chlorophyll metabolites. The chlorophyll profile found in the livers of mice fed a chlorophyll-rich diet showed that the formation and/or absorption of pheophorbides, pyro-derivatives and phytyl-chlorin require the occurrence of a precise first-pass metabolism. In addition, the apical absorption of pheophorbide- rich micelles was significantly inhibited in Caco-2 cells pre-incubated with BLT1.

Conclusion

Pheophorbide absorption is, at least partly, protein-mediated through SR-BI. This active absorption process could explain the specific accumulation of pheophorbide in the livers of animals fed a chlorophyll-rich diet. A complementary mechanism could be the de-esterification of pheophytin in the liver, yielding pheophorbide and phytol, which would explain the origin of phytol in the liver. Hence, our results suggest two molecular mechanisms responsible for the accumulation of the health-promoting compounds pheophorbide and phytol.
1. Introduction

Spirulina is commercialized as a “health food” recognized by the WHO and the FAO.[1] A rich source of biologically active ingredients and nutrients – proteins, lipids, phycobilins and carotenoids – spirulina also contains a relatively high amount of chlorophylls. This group of pigments is the most ubiquitous in nature as they play a key role in photosynthesis. Significant functions of chlorophylls have also been evidenced in animals. Indeed, it can be anticipated that after millions of years of close interaction through diet, the animal body has found some way to take advantage of the physicochemical characteristics of chlorophylls. It is known that consumption of fruits and vegetables is associated with a lower risk of cancer and that they may be protective against the development of several other chronic diseases [2]. In particular, numerous scientific studies have shown that chlorophylls and their derivatives from foods develop benefits to human health when consumed [3], through antioxidant, antimutagenic [4] and antigenotoxic action activities [5]. Specifically, research has focused on the chemo-preventive effects in humans [6], with evidence from numerous in vivo [7] and in vitro [8] studies.

Despite the extensive literature pointing to the health benefits derived from the intake of a diet rich in chlorophyll pigments, our understanding of the first-pass metabolism and subsequent systemic assimilation of chlorophylls is scarce [8]. This is particularly remarkable considering that green vegetables are present in our daily dietary patterns and their intake is actively promoted by international health agencies through campaigns like 5 A Day [3]. This lack of understanding about the biochemical changes of chlorophyll pigments that take place during digestion, and their bioaccessibility and metabolism has restrained the interest in chlorophylls as bioactive compounds. Preliminary research on the assimilation of chlorophylls was based on the quantification of chlorophylls derivatives in human and animal feces, assuming that they were not absorbed in the organism [9] as almost all the ingested chlorophylls were excreted. More recently, Motta-Fernandes, Gomes, and Lanfer-Márquez,[10] applied the same balance mass (determining ingested
against excreted) chlorophylls and estimated the apparent absorption of 3.4% of chlorophylls from spinach. Recently, it has been published [11] an organ-specific distribution of chlorophyll-related compounds from dietary spinach in rabbits. But the uncertain chlorophyll identification, the lack of control samples and the presence of chlorophyll derivatives at time 0 due to an existing "green diet" before the beginning of the experiment meant that conclusions were ambiguous. Indeed, to date, the only convincing in vivo evidence of the absorption and accumulation of chlorophyll structures in mammals is related to copper-chlorophyllins [12]. This work described the presence of copper-chlorin e₄ ethyl ester in the sera of individuals participating in a chemoprevention trial after the ingestion of copper-chlorophyllins. Moreover, a later study estimated that dietary sodium copper-chlorophyllins accumulated in different organs of Wistar rats [13]. However, these compounds arise from a chemical process (chemical hydrolysis plus addition of copper salt) applied to natural chlorophyll extracts. Consequently, copper-chlorophyllins should not be denoted as natural pigments [14] as they are not found in raw fruits, vegetables or seaweeds. Sodium copper-chlorophyllins are synthetic compounds that are commercially available as food colorants, and their absorption/accumulation is unlikely to represent the in vivo absorption/accumulation of the natural chlorophylls present in fruit and vegetables.

In this sense, only in vitro experiments have been performed to obtain information on the digestion, absorption, and metabolism of natural chlorophyll pigments from food sources. Ferruzzi, Failla and Schwartz,[15] applied an in vitro protocol of digestion and estimated the subsequent uptake of chlorophylls from the micellar fraction by the Caco-2 cellular model. The native chlorophylls from spinach were transformed to Mg-free pheophytin-type derivatives during digestion, and 5–10% of the micellar pheophytin-type derivatives accumulated in the cells, suggesting that chlorophyll derivatives could be absorbed and eventually transported to peripheral tissues. Subsequently, these results were confirmed, applying the same in vitro protocol to pea preparations [16], standards of chlorophyll derivatives isolated from natural sources [17], and seaweeds [18-19]. Specifically, it has
been shown in vitro that de-phytylated chlorophylls (pheophorbides) are preferentially absorbed over phytated chlorophylls (pheophytins) [17-19]. Through comparative experiments with the Caco-2 cellular model, it has been hypothesized that pheophytin a at different concentration levels in the cell culture medium is absorbed by passive diffusion, while pheophorbide is absorbed by a combination of two mechanisms. The authors claimed that the saturation of the absorption rate observed at concentrations below 0.5 µM is compatible with a passive transport mechanism. The linear absorption rate obtained for the 0.5-1.5 µM concentration range points to a simple diffusion mechanism. However, these experiments were developed with the aqueous micellar fraction (AMF) of pheophorbide a standard obtained after the application of an in vitro digestion protocol; thus the composition and concentration of the AMF is limited by the methodology, which does not allow the pigment to reach physiological concentrations in the AMF. The application of synthetic mixed micelles for related lipophilic compounds such as carotenoids [20-22] has allowed physiological micellar concentrations (7µM) to be reached. Consequently, it is possible to analyze the intestinal absorption process of chlorophyll derivatives with this technique at a concentration similar to the physiological one (ca. 2-5 µM, [12]).

In summary in vivo evidence of the absorption/accumulation of natural chlorophylls from food sources is lacking. The aim of this study was to determine whether natural chlorophylls from a dietary source are metabolized and accumulated by mammals in vivo, as well as examine the possible implication of protein-type membrane transporters of chlorophyll metabolites. Therefore, the diet of a mammalian model (mice) was supplemented with a common source of chlorophyll, spirulina, and the first-pass metabolism of chlorophylls determined.

2. Materials and methods

2.1 Chemicals and standards

Solvents for liquid chromatography were purchased from Teknokroma (Barcelona, Spain), while PAR grade and LC/MS grade solvents and water were supplied by Panreac (Barcelona, Spain). The
deionized water was obtained from a Milli-Q 50 system (Millipore, Milford, USA). Standard of chlorophyll and pheophorbide a were purchased from Wako (Neuss, Germany) and chlorin e₆ from Frontier Scientific (Logan, USA). The other chlorophyll derivatives were obtained following previously detailed the protocols [23-24]. Commercial Spirulina (NaturGreen) was purchased in a local store. N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO, purity > 99.9%), sodium taurocholate, tetrabutylammonium acetate and ammonium acetate (purity > 98%), 2-hexyl-1-cyclopentanone thiosemicarbazon (BLT1), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-sn-glycero-3-phosphocholine (lysophosphatidylcholine), mono-olein, free cholesterol, oleic acid, sodium taurocholate, trypsin (500 BAEE units porcine trypsin), fetal bovine serum, penicillin, streptomycin, L-glutamine, nonessential amino acids, Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L glucose, and phosphate-buffered saline (PBS) were provided by Sigma-Aldrich Chemical Co. (Madrid, Spain).

2.2 Experimental section

Rodent-pellets (Harland Teklad) were provided by ssniff-Spezialdiäten (Soest, Germany). To formulate the feed supplemented with chlorophyll pigments, grinded pellets were mixed with spirulina powder (15% w/w). Water was added to improve the homogenization of the feed and recreate its original pellet-shape. The supplemented pellets were dried in an oven at 30°C for 24 h to reestablish their hardness and avoid spoilage during storage of the feed. After drying, the supplemented pellets were vacuum packed and stored at -20 °C.

2.2.1 Animal model

C57BL/6N mice were obtained from the Animal Production and Experimentation Service (Seville University, Spain). 3 months old male mice (ca. 40 g) were housed in conventional cages (3-4 animals per cage). The mice were kept on a standard rodent diet and water ad libitum for two weeks. Then, a group of 30 mice received the feed supplemented with Spirulina, while 10 mice
continued with the standard diet (control group). At week 2, 5 mice of the control group and 15 mice from the supplemented feed group were euthanized by cervical dislocation. The livers were excised, weighed, rinsed with 0.9% saline solution and then stored at -80 ºC until analysis within 1 week. Fecal samples were collected from each group of mice and stored at -80 ºC until analysis within 1 week. At week 4, the same protocol was applied to the rest of the mice in both groups. The use of the animals for this experiment and the protocols were carried out according to the European Union directive (2010/63/EU) for the use of laboratory animals and were approved by the local Ethical Committee (study number 0158-N-15, Junta de Andalucía, Spain).

2.2.2 Culture of Caco-2 cells.

Caco-2 (Caucasian colon adenocarcinoma) cells were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain) and stocks were maintained in complete medium as described previously [19]. At 70%-80% confluency, the cells were sub-cultured with trypsin and seeded in 75 cm² flasks (Nunclon-treated surface, Nunc A/S), at densities of 3 ×10⁴ cells/cm², following published protocols [19]. All the experiments used highly differentiated monolayers around 11-14 days after reaching confluency. Medium was replaced every 2-3 days, and the last replacement of medium before the experiments was carried out using serum-free medium.

2.2.3 Preparation of pheophorbide a-rich micelles

For the delivery of pheophorbide a to cells, mixed micelles were prepared as described before. [20] to mimic the physiological micellar concentration with slight modifications. Appropriate volumes of the micellar compounds were transferred to glass bottles to obtain the following final concentrations: 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.3 mM monolein, 0.1 mM free cholesterol, 0.5 mM oleic acid. Stock solution solvents were carefully evaporated under nitrogen. The dried residue was dissolved in DMEM containing 5 mM taurocholate and pheophorbide a (2.5 μM), and vigorously mixed by sonication at 720W for 3 min. Pheophorbide a was dissolved in acetone and the addition of acetone volume is 1% (v/v). The
mixtures obtained were sterilized and filtered by passing them through a sterilized 0.20 μm filter (Acrodisc HT Tuffryn membrane). HPLC analysis showed a few amount of $^{13}\text{OH}$ pheophorbide (less than 5%) yielded after this process, and the pigment in micelles presented the normal absorption spectrum as before. Micelles were freshly prepared before the incubation with Caco-2 cells, as well as the measurement of the pheophorbide $a$ concentration in the micellar preparations. The size of micelles was evaluated with Malvern Zetasizer (Malvern, UK).

2.2.4 Cytotoxicity assay of organic solvent and BLT1

Cytotoxicity assay was carried out on a 96-well plate according to the methods of Reboul et al. [20] to control the addition of exogenous chemicals including acetone in the preparation of mixed micelles, DMSO and BLT1 in the micelles for inhibitory assay. Cytotoxicity was assessed by a CellTiter 96 Aqueous One Solution assay (Promega, Madison, USA). Cells were seeded at a density of 10000 cells per well, and grown for 5 days. Then cells received either acetone ranging from 0.1% to 2% (v/v), or DMSO, 0.1% to 0.5% (v/v) in DMEM for 3 h and the results showed acetone and DMSO were not toxic in the tested range for Caco-2 cells. Latter, the same experiment was conducted to assay BLT1 cytotoxicity (0 ~ 25 μM, in 0.2% DMSO) and results showed BLT1 was not toxic up to 15 μM for Caco-2 cells.

2.2.5 Delivery of pheophorbide $a$ rich micelles to Caco-2 cells

The analytical conditions used have been described previously [25]. Before the incubation experiment, cell monolayers were washed twice with 155 mL of PBS to eliminate interfering compounds. Cell monolayers were incubated at 37 °C for 5 hours with 15 mL of the micelles. After the incubation period, media from apical side of the membrane were harvested and monolayers were washed twice with 10 mL of ice-cold PBS containing 10 mM sodium taurocholate to eliminate adhered pigments, then scraped and collected in 6 mL of PBS containing 10% ethanol (v/v). All the samples were stored at -23°C under nitrogen before extraction and HPLC analysis. Aliquots (20 μL) of cell samples were used to estimate protein concentrations.
The amount of protein in cell suspension was determined by bicinchoninic acid (BCA) assay (Sigma-Aldrich Chemical Co., Madrid, Spain). Cell lysates and protein standards (bovine serum albumin) were separately mixed with 200 μL mixture of BCA solution and 4% CuSO4 and heated at 60 °C for 15 min. The absorbance at 562 nm was measured with a 96-well plates reader (Imark, Bio-Rad).

Methods were described previously [20]. Briefly, cell monolayers were washed twice with 10 mL of PBS and the effect of inhibitor (BLT1) on pheophorbide a transport was evaluated by first monolayer pretreatment with 15 mL of either DMSO (control, 0.2%, v/v) or BLT1 at different concentrations ranging from 0.1 to 10 μM for 1 h, and then cells received pheophorbide a micelles with BLT1 at the pre-incubated concentration.

All procedures were performed under dimmed green light to avoid any photooxidative process of the chlorophyll derivatives. Pellets (both standard and supplemented ones), livers, or feces were homogenized with 25 mL of N, N - dimethylformamide saturated with MgCO3 [24] and vacuum filtered. The solid was collected and extracted twice with the same conditions. The filtrates were combined in a decanting funnel and mixed with 100 mL of diethyl ether and 100 mL of NaCl solution 10% (w/v). The mixture was stirred and allowed to stand until complete separation of the organic layer. The lower phase was discarded and the organic layer was washed twice with 100 mL of Na2SO4 solution 2% (w/v), and finally filtered through a solid bed of 50 g anhydrous Na2SO4. The filtrate was concentrated in a vacuum rotary evaporator to dryness at 30º C and the dry residue was solved in 0.5 mL (liver sample) or 1 mL (pellet or fecal sample) of acetone. The protocol for chlorophyll extraction from Caco-2 cells has been detailed previously [19] and briefly consists on successive extractions with ethanol (0.1% BHT), acetone (0.2% BHT), diethyl ether and 10% NaCl.
Extraction process was repeated for at least three times as above and the dried residue after evaporation was dissolved in 0.25 mL of acetone. All the samples were filtered through a 0.22 µm nylon filter and stored at -20°C until analysis within 1 week.

2.2.9 Sample analysis by HPLC-ESI/APCI-hrTOF-MS

The liquid chromatograph was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed as described before [23-24]. A stainless steel column (200×4.6 mm, 3 µm particle size) packed with Mediterranea Sea18 (Teknokroma, Barcelona, Spain) was used. The injection volume was 30 µL and the flow rate was 1 mL/min, and a split post-column of 0.4 mL/min was introduced directly on the mass spectrometer ion source. Mass spectrometry was performed using a micrOTOF-QII™ High Resolution Time-of-Flight mass spectrometer (UHR-qTOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an ESI and APCI source. The instrument was operated in positive ion mode using a scan range of m/z 50-1200 Da. Mass spectra were acquired through broad-band collision induced dissociation mode, providing simultaneously MS and MS/MS spectra. The instrument control was performed using Bruker Daltonics HyStar 3.2. Data evaluation was performed with Bruker Daltonics DataAnalysis 4.1 as described previously [23-24]. From the HPLC/qTOF-MS acquisition data, an automated peak detection on the EICs expected for the [M+H]+ ion of each compound in the database was performed with Bruker Daltonics TargetAnalysis™ 1.2 software. The software performed the identification automatically according to mass accuracy and in combination with the isotopic pattern in the SigmaFit™ algorithm [26]. Only those hits with mass accuracy and SigmaFit values within the tolerance limits, which were set at 5 ppm and 50, respectively are considered as positive matches. The interpretation of the MS/MS spectra was performed using the SmartFormula3D™ module included in the DataAnalysis software [26].

2.2.10 Pigment quantification by HPLC-UV-Visible
The identified chlorophyll derivatives were quantified by reversed-phase HPLC using a Hewlett-Packard HP 1100 liquid chromatograph. A Mediterranea Sea18 column (200×4.6 mm, 3 μm particle size) was used (Teknokroma, Barcelona, Spain) protected by a guard column (10×4.6 mm) packed with the same material. Separation was attained using the elution gradient described before [23].

The on-line UV-Visible spectra were recorded from 350 to 800 nm with the photodiode–array detector, and sequential detection was performed at 410, 430, 450 and 666 nm. Data were collected and processed with a LC HP ChemStation (Rev.A.05.04). Quantification of pigments was achieved with the corresponding calibration curves (amount versus integrated peak area). The calibration equations were obtained by least-squares linear regression analysis over a concentration range according to the observed levels of these pigments in the samples. Triplicate injections were made for five different volumes of each standard solution.

2.2.11 Statistical analysis

The aim of the study was to observe accumulation of chlorophyll derivatives in the group of supplemented feed of mice. Although the standard rodent-diet does not contain any trace of chlorophyll derivatives, and diet is the single origin of these compounds in animals, a control group was included to take into account whether the supplemented feed introduced any change in the dietary habits and physical performance of the mice. Thus, it was observed that the supplemented feed group of mice did not reject their feed and they followed their normal habits and behavior patterns with similar consumption rates to the control group. In any case, samples were obtained from the control group to clearly show that they did not accumulate any trace of chlorophyll derivatives. The size of the supplemented feed group of mice was calculated by power analysis considering the statistical test to be applied for comparison of data (t-test), the significance level that was set at P<0.05 with a statistical power of 0.90, and the standard deviation values from similar studies [27]. The principles of the 3Rs were applied in the experimental design and the protocols applied in this study.
Each absorption experiment was repeated at least three times and the absorption rate of pheophorbide \(a\) was expressed as \(\text{pmol of pigment absorbed} \times \text{min}^{-1} (\text{mg of protein})^{-1}\). For the calculation of absorption percentage in inhibitor experiment, absorption percentage means nanograms of pheophorbide \(a\) absorbed in pretreated monolayers \(\times \) 100/ nanograms absorbed in the control (without inhibitor treatment).

Differences were compared by one-way analysis of variance (ANOVA) using Statistica for Windows (version 6, StatSoft, Inc., 2001) with student\'s t-test as post hoc comparison test. Differences were considered significant at \(P < 0.05\).

3. Results

3.1 Identification of chlorophyll pigments

Figure S1, Supporting Information depicts the structures identified in the samples analyzed in this study. Only a series chlorophyll pigments (methyl group at C7) were observed in the samples analyzed in this study, in accordance with the typical profile of the chlorophyll source used for supplementation (spirulina) that belongs to the cyanobacteria phylum. In addition to chlorophyll \(a\) (the main parent compound with a characteristic Mg\(^{+2}\) central ion and the C17\(^3\) position esterified with phytol chain, \(R_3\) ) some derivatives were identified in the diet material, including chlorophyllide (derivative preserving the Mg\(^{+2}\), but de-esterified at the C17\(^3\) position), pheophytin (the de-metallo-chlorophyll derivative featuring 2H\(^+\) in the core of the tetrapyrrolic assembling, but retaining the esterification with a phytol chain) and pheophorbide (de-metallo-chlorophyll derivative, also de-esterified at the C17\(^3\) position). Allomerized products (the 13\(^2\)-hydroxy- and the 15\(^1\)-hydroxy-lactone-derivatives) were also observed, arising from oxidative reactions at the fifth isocyclic ring of the before mentioned compounds. Finally, the pyro-derivatives which are produced after the decarboxymethylation reaction at C13\(^2\) position were also identified.

These products were identified according to their chromatographic behavior (retention time), UV-Visible spectra, and MS high-accuracy measurements, including mass error, isotopic pattern and the
featured product ions. These characteristic data, presented in Table S1, Supporting Information, were compared with those of pure standards and data available in the literature [15, 23-24]. However, the identification of phytly-chlorin e₆ was supported by the chromatographic behavior and features of the UV-visible spectrum in comparison with its de-esterified counterpart chlorin e₆ because this is the commercial standard available [28]. The MS measurements described for phytly-chlorin e₆ are the first experimental data reported for this chlorophyll derivative.

3.2 Quantitative profile of chlorophyll derivatives in the diet

The diet of mice in the control group did not contain any chlorophyll pigments. Thus, it is evident that the control group did not ingest any chlorophyll derivatives. On the contrary, fresh prepared spirulina pellets mainly contain chlorophyll a and its epimer (peaks 12 and 13, Figure 1a), a chlorophyll profile typical of cyanobacteria. However, during the slow drying process (30° C for 24 h), different chlorophyll derivatives were produced (Figure 1b). The drying process was necessary to obtain the required consistency of the spirulina and feed mix, to avoid spoilage of the final product, and produce a feed with the representative pattern of frequently ingested chlorophylls [29]. Thus, the mice in the experimental group were fed a dried chlorophyll-supplemented feed with a chlorophyll profile consisting of 19% intact chlorophyll a, ca. 7% of pheophorbide-type derivatives and 74% of pheophytin-type derivatives (Table 1). The allomerized products reached 4% of the total chlorophyll profile and the pyro-derivatives 17%. The total amount of chlorophyll pigments in the dry mixture of spirulina-supplemented feed was 1.5 g/kg, an amount predicted to be close to the quantity obtained from the regular dietary intake of vegetables [30]. Spinach, broccoli, rucula, kale, lettuce, green pepper, avocado and peas [14] can provide even higher amounts of chlorophylls in the Western diet.

3.3 Accumulation of chlorophyll derivatives in the liver

The livers of mice fed with the spirulina-supplemented diet accumulated chlorophyll derivatives (Figure 2a). In contrast, the livers of mice fed the control diet did not accumulate any chlorophyll
derivatives (Figure 2a, insert). Mice were fed for 1 month with the feed supplemented with Spirulina as the source of chlorophyll derivatives. After two weeks of supplementation, the total content of chlorophyll derivatives in the tissues reached a maximum value and then plateaued until the end of the experiment (Table 1), with no significant differences observed between 2 and 4 weeks (P>0.05). The chlorophyll a (and chlorophyllide a to a lesser extent) present in the spirulina feed was not detected in the liver. Chlorophyll a is prone to transformation processes, including demetallation and allomerization, as observed in simulated in vitro digestion [15], yielding the corresponding pheophytin, 13\textsuperscript{2}-OH and 15\textsuperscript{1}-OH-lactone-type derivatives. While the pheophytin-type derivatives continued as the major contributors to the pigment profile in the livers of spirulina-fed mice (71\% of the total chlorophyll content), pyro-derivatives, allomerized products, and pheophorbide-type derivatives increased their presence in the chlorophyll pattern accumulated in that tissue. Thus, pyro-derivatives increased from a 17\% representation in the supplemented feed to ca. 43\% of the total chlorophyll derivatives present in the liver. Likewise, pheophorbide-types increased from ca. 7\% in the supplemented feed to almost 28\% of the total chlorophyll pigments detected in the liver (P<0.05), and allomerized products increased significantly from 4\% in the feed to 22\% in the liver.

The identification of phytyl-chlorin e\textsubscript{6} in the livers of the supplemented group of mice is of particular note because no other chlorophyll derivatives observed in the tissue lack the integrity of the macrocycle assembly (structure C, Figure S1, Supporting Information). This study reports for the first time the MS characteristics of phytyl-chlorin e\textsubscript{6} (Table S1, Supporting Information) and its presence in an animal tissue after dietary supplementation with a chlorophyll source. Phytyl-chlorin e\textsubscript{6} presents a main product ion (m/z 597.2710 Da) corresponding to the loss of the phytyl chain, the principal fragmentation in phytylated chlorophylls [23].

3.4 Effect of SR-BI inhibitor on pheophorbide a uptake by Caco-2 monolayers
Livers from animals with a chlorophyll-supplemented diet had a significant accumulation of pheophorbide a. Pheophorbide a could be transported into the liver through a protein-type carrier, although the molecular nature of the possible transporters implied in chlorophyll absorption are currently unknown. Carotenoids, similar lipophilic compounds, are taken up by enterocytes through several apical protein-type membrane transporters, which have been shown to facilitate carotenoid uptake [20-22]. Although few proteins have been proposed as mediators in the cell absorption of carotenoids, the scavenger receptor B type I (SR-BI/SCARB) is involved in the transport of different carotenoids (lutein, β-carotene, α-carotene, β-cryptoxanthin and lutein) in diverse animal tissues (intestinal and retinal) [20-22]. Most of these results were obtained from experiments employing blocks lipid transport 1 (BLT1), a specific inhibition of transport by SR-BI. We used a similar strategy to check the possible implication of SR-BI in the intestinal absorption of pheophorbide a. We report here for the first time the production of pheophorbide a-rich micelles, which were delivered to the apical medium of Caco-2 culture at an absorption rate of 2.16 pmol/min/mg protein (Table 2). Pre-incubations of the cell monolayers with different amounts of BLT1 significantly inhibited (P<0.05) the absorption of pheophorbide a.

3.5 Quantification and distribution of chlorophyll derivatives in feces

The chlorophyll profile of the feces of the supplemented group of mice was similar to the chlorophyll pattern observed in the feed they ingested, with the exception of chlorophyll a and chlorophyllide-type derivatives that were almost completely transformed during digestion into other chlorophyll derivatives (Table 1). Surprisingly, some intact chlorophyll a was detected in the feces. The percentage value of the pheophorbide-type derivatives in the feces (8%) correlates well with the value observed in the supplemented feed (ca. 7%). Indeed, the percentage value of allomerized derivatives in the feces (6%) also resembles that of the supplemented feed (4%). This steady trend was not observed for the content of pheophytin-type and pyro-derivatives, which had an increased
presence in the chlorophyll profile of the feces, reaching 91% and 42% of the total chlorophylls, compared to starting values of 74% and 17% in the supplemented feed, respectively.

4. Discussion

Identification of chlorophyll derivatives in the samples was performed with the analysis of the chromatographic behavior, features of the UV-visible spectrum and characteristics of the mass spectra, including isotopic mass values, mass error and isotope pattern of both the protonated ion $[\text{M+H}]^+$ and the corresponding product ions obtained after MS-fragmentation reactions of the former. Acquisition of mass spectra was made with an ESI source for the de-phytylated chlorophyll derivatives while the ionization of the phytlated ones was made with an APCI source. The use of different ionization sources for polar and apolar chlorophyll derivatives was applied to obtain higher signal records and to increase the resolution of the mass spectra, as it has been shown before [23-24]. A considerable change of the chlorophyll profile was observed from the raw material (spirulina, Figure 1a) to the processed supplemented feed (Figure 1b). This difference should be noted as the common transformation course that the parent chlorophyll structure follows during the in vivo metabolism of photosynthetic tissues [31], or to the storage time or temperature applied for processing [32]. The parent chlorophyll is prone to transformation reactions [33], and thus chlorophyll metabolites/derivatives, such as pheophytin, pheophorbide and their allomerized products, are observed in fresh green vegetables and seaweeds, and their presence increases during cooking, microwaving, pasteurization of canned vegetables, production of vegetable oils, and through storage [29]. Indeed, most of the evidence supporting the health benefits of chlorophyll derivatives has been examined with pheophorbide- and pheophytin-type derivatives because they are the chlorophyll derivatives present in our regular diet. When fresh chlorophyll sources are ingested, the parent chlorophyll structure is completely transformed into pheophytin-types derivatives during digestion as it will be shown below. Pyro-derivatives are also commonly present in our diet [29], and they are produced following the application of higher temperature regimes, like
those applied for the refining of vegetable oils, or a longer storage period, both of which influence the progress of the decarboxymethylation reaction [34].

The pH conditions reached in the stomach and the enzymatic reactions taking place there and later in the small intestine conform the perfect environment to transform the original chlorophyll into pheophytin-type derivatives, as observed in in vitro studies [15-18]. Almost all the native chlorophyll content is transformed in the stomach through pheophytinization, although a small fraction of intact chlorophyll $a$ was still present in the feces of mice. Additionally, the corresponding allomerized products of pheophytin increased their presence through enzymatic reactions [16] and chemical processes [17] that take place during digestion. However, there seems to be no reaction yielding pheophorbide-type derivatives or pyro-derivatives. Actually, the standards of pheophytin, pheophorbide and its pyro-derivatives are stable during in vitro digestion and no transformation was observed in their structures during that process [17]. The subsequent absorption of chlorophyll derivatives has been studied in vitro using the Caco-2 cell model [15-17, 19]. Using this model, which simulates the absorption processes of the intestinal epithelium, the uptake of in vitro digested chlorophyll seems to be selective for pheophorbide $a$ and pyropheophorbide $a$, showing similar absorption rates. However, an in vivo observation of this selective absorption had not been presented until now. Our results confirm that the majority of parent chlorophyll $a$ does not reach the intestinal epithelium, considering the pH conditions of the stomach during digestion and the straightforward progress of the pheophytinization reaction. The initial chlorophyll $a$ fraction was transformed into pheophytin $a$ and its allomerized products (Figure S1, Supporting Information). Surprisingly, a small amount of chlorophyll $a$ maintained its structure during digestion and was eliminated intact in the feces. Consequently, the significance of pheophytin-type derivatives in the chlorophyll profile after digestion, and potentially available for absorption, is even higher than the 74% value observed in the supplemented feed (Table 1).

Assuming that the complete chlorophyll $a$ content in the supplemented feed is transformed into
pheophytin-type derivatives, their percentage value increased to 92%, a number close to the one observed in the feces (91%, Table 1). Therefore, we consider that pheophytin-type derivatives represent the chlorophyll profile available for absorption in the intestinal epithelium. However, although pheophytin-type derivatives were the major chlorophylls present in livers, their value declined significantly in comparison to the initial amount in the feed, while the pyro-derivatives and the pheophorbide-types, as well as the allomerized products significantly increased their presence in the distribution pattern of chlorophyll derivatives of the livers. It is worth restating that no exchange of pheophytin-type derivatives to pyro-derivatives or pheophorbide-type derivatives takes place during digestion, taking into account the results observed from in vitro studies. Only the conversion of pheophytin a into its allomerized products has been shown to take place to some extent during digestion. In any case, the total chlorophyll derivatives content in the liver reached a plateau after two weeks of supplementation. In humans, the steady state of copper chlorophyllins was reached at 8 weeks [12].

Therefore, the increases observed for pheophorbide-type derivatives in the chlorophyll pattern of the livers could be attributed to a preferential absorption of these derivatives or their metabolic conversion from pheophytins in the liver. The former mechanism is supported by the results obtained with the specific inhibitor of SR-BI, BLT1. BLT1 was selected because it inhibits lipid transport by SR-BI in nanomolar concentrations and it does not cross-react with other protein-type transporters [20]. The finding that the absorption of pheophorbide a was significantly decreased when cells were treated with BLT1 strongly suggests that SR-BI is involved in the transport of pheophorbide a. The fact that increasing amounts of BLT1 were unable to completely inhibit the absorption of pheophorbide a could suggest that additional absorption routes might be involved. This is characteristic in carotenoid absorption, for which inhibition with specific inhibitors or antibodies is not complete [20-22]. Thus, we consider that the preferential absorption of pheophorbide a is the most plausible mechanism explaining this result. To the best of our
knowledge, there is no research related with the identification of transporters implicated in chlorophyll compounds absorption. Only Jonker et al.,[5], indirectly and in vitro, identified the breast resistance protein, an intestine ABC transporter, as a reasonable candidate for a pheophorbide $a$ efflux-transporter. Nevertheless, we cannot exclude the possibility that an additional mechanism could help in part in the significant in vivo accumulation of pheophorbide $a$ in the liver, such as the de-esterification of the phytyl chain from accumulated pheophytin. Several esterase-type enzymes have been described in liver [35] that may perform this reaction. Indeed, phytol metabolites present in animal tissues are exclusively derived from the phytol side chain of chlorophylls. As a consequence of the consumption of large quantities of grass, ruminant animals contain high amounts of phytol derivatives. In the organism, phytol can be metabolized in the liver to phytanic acid, phytanic acid and pristanic acid [36]. However, the intriguing question is how the phytol reaches the liver. The possibility that pheophytin could be effectively de-esterified in the liver would explain the molecular mechanism of the origin of the phytol present in the liver.

Independently of the active mechanism, the in vivo accumulation of pheophorbide $a$ in the liver is a hint at the existence of a specific mechanism of chlorophyll metabolism in animals. The significant increase of pyro-derivatives in the pool of chlorophyll derivatives accumulated in the livers cannot be explained by enzymatic hydrolysis from their parent chlorophyll derivatives as no decarboxymethylase activity has been described in the liver to date. Gandul-Rojas et al.[17] reported the preferential in vitro absorption of pyropheophorbide $a$ but not of pyropheophytin $a$.

Therefore, it would be interesting to investigate the role of the protein-type transporters involved in the accumulation of pyro-derivatives in future studies. On the other hand, the increase of pyro-derivatives observed in the feces raises further questions. As previously noted, no transformation process of pheophytin or pheophorbide into pyro-derivatives exists, at least in the small intestine. Thus the increased proportion of pyro-derivatives in the feces compared to the chlorophyll profile of the supplemented feed could be attributed to conversion processes specifically available in the
colon. Ferruzzi et al.,[15] showed that intestinal microflora might be involved in the interconversion of chlorophyll derivatives in the gut. Another plausible alternative is the activity of efflux transporters in the colon that excrete the accumulated pyro-derivatives since we observed similar values of pyro-derivatives in the liver and in the feces. The protein-type transporters mentioned above, breast cancer resistance protein and bilitranslocase-like protein could be candidates for the excretion of accumulated pyro-derivatives. In any case, it is important to highlight that a chlorophyll structure similar to pyro-derivatives obtained from the diet can be metabolized and utilized as a visual pigment by the dragon fish [37]. Finally, the interest in pyro-derivative structures has been recently expanded due to their application as photosensitizers in photodynamic therapy (PDT) [38]. Therefore, our results showing the accumulation of these compounds in the liver may point to specific treatment of cancer by PDT in that tissue. Up to now, treatment with photosensitizers is administered intravenously. The results obtained in the present work emphasize the importance of the diet during cancer treatment, as enhancers of a specific therapy or an alternative for photosensitizer administration.

The identification of phytyl-chlorin $e_6$ in the livers of chlorophyll-supplemented mice introduces a divergence from the results of \textit{in vitro} studies (which lack the portal vein and hepatic system) on the digestion and intestinal absorption of chlorophyll pigments in animals. Thus, it is assumed that the structural modifications and interconversions of chlorophyll derivatives exclusively affect the peripheral positions leaving the macrocycle intact. Phytlyl-chlorin $e_6$ lacks the isocyclic ring (structure C, Figure S1, Supporting Information), while two carboxylic acid groups appear at the C13 and C15 positions. The opening of the isocyclic ring to originate chlorins, rhodins or purpurins is traditionally associated with chemical reactions such as saponification [39]. Although the accumulation of this compound in livers was secondary in comparison with the rest of chlorophyll derivatives, its presence indicates the existence of alternative metabolic pathways that modify the macrocycle structure. The detection of the phytylated form of chlorin $e_6$ points to
pheophytin-type derivatives as its precursors, because pheophorbides are dephytylated structures.

Consequently, this kind of metabolic transformation probably increases the polarity of the parent compound, facilitating further metabolism and/or excretion. The fate of these kinds of metabolites is unknown so far, although some information exists regarding the use of chlorin e₆ derivatives in photodynamic therapy. For example, several studies related to the development of “second generation” photosensitizers based on the chlorin e₆ structure [40] have dealt with their tissue distribution. These studies implied the intravenous administration of chlorin e₆ photosensitizers and the posterior localization of these compounds in the liver and other tissues [41]. In addition, the presence of chlorin e₆ in the retina of several animals have been shown to enhance the red vision [42-43]. However, our data reveal Table 1) that a chlorin e₆ counterpart is accumulated in liver because of the metabolism of parent derivatives through processes that modify the macrocycle, rather than through intravenous administration, an unprecedented result in the literature.

5. Conclusions

Recent discoveries have shown the specific functions of several chlorophyll metabolites of developing in animal tissues, including the production of ATP [44], the regulation of the redox status in plasma [45], implications as visual pigments [37, 42-43], and activation of specific nuclear receptors that could control lipid abnormalities in common diseases such as obesity, diabetes, and hyperlipidemia [46] among others. Evidently, the origin of chlorophyll metabolites present in the animal body is circumscribed to the diet. In humans, the ingestion of chlorophyll derivatives is estimated at around 25-85 mg/day [47], but knowledge on the fate of derivatives in the organism are lacking. Our results show the existence of a first-pass metabolism of chlorophylls. We have demonstrated in vitro that the transport of pheophorbide a is, at least partly, facilitated by the scavenger receptor SR-BI. Further research is required to elucidate the molecular mechanisms implicated in the absorption and metabolism of chlorophylls in mammals. Our study highlights that apolar chlorophyll derivatives, i.e. those retaining the phytly chain in the structure, are available for
absorption from a dietary source and accumulate in the liver where they can be further metabolized to provide a source of phytol.

6. References


[27] Festing, M.F., Altman, D.G., Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. 2002, 43, 244-258.


**Figure legends**

Figure 1: HPLC chromatograms at 660 nm of chlorophyll compounds in fresh Spirulina pellets (a) and in dried Spirulina pellets (b). Peak numbers as in Table S1, Supporting Information.

Figure 2: HPLC chromatograms at 660 nm of chlorophyll compound in biological samples: liver (a) and feces (b). Inserts correspond to control diets. Peak numbers as in Table S1, Supporting Information.

**Author contributions**

I.V., K.C., I.B., performed the experiments; I.V., J.R., A.P., M.R. analyzed and interpreted the data; A.P., M.R., conceived the project, designed the study and supervised the research.; I.V., A.P., M.R. wrote the manuscript.

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Conflict of interest

All the authors declare that they have no conflict of interest.
Table 1: Chlorophyll derivatives identified and quantified in pellets and biological samples of spirulina fed mice (mg/kg d.w. ± SD). Chlorophylls, pheophorbides, pheophytins and chlorins accounts for the 100% of total chlorophylls. The chemical modification responsible of the pyro-derivatives or allomerized (13²-hydroxy and 15¹-hydroxy-lactone) chlorophylls impacts to pheophorbides and pheophytins amounts (Figure S1, Supporting Information).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pellets</th>
<th>Liver 2 weeks</th>
<th>Liver 4 weeks</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyllide a</td>
<td>6.90 ± 0.77</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15¹-Hydroxy-lactone pheophorbide</td>
<td>1.16 ± 0.04</td>
<td>0.42 ± 0.03</td>
<td>0.42 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>13²-Hydroxy-pheophorbide a</td>
<td>1.93 ± 0.22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pheophorbide a</td>
<td>48.52 ± 4.38</td>
<td>1.30 ± 0.16</td>
<td>1.62 ± 0.02</td>
<td>26.22 ± 0.17</td>
</tr>
<tr>
<td>Pheophorbide a’</td>
<td>-</td>
<td>0.60 ± 0.02</td>
<td>0.80 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>13²-Hydroxy-pheophorbide a</td>
<td>8.09 ± 0.46</td>
<td>0.44 ± 0.07</td>
<td>0.51 ± 0.08</td>
<td>9.40 ± 0.07</td>
</tr>
<tr>
<td>Pyropheophorbide a</td>
<td>33.85 ± 1.79</td>
<td>2.41 ± 2.01</td>
<td>2.86 ± 1.91</td>
<td>77.70 ± 5.42</td>
</tr>
<tr>
<td>Pyropheophorbide a’</td>
<td>6.17 ± 0.41</td>
<td>0.47 ± 0.05</td>
<td>0.44 ± 0.05</td>
<td>12.65 ± 0.95</td>
</tr>
<tr>
<td>Phytyl chlorin e₆</td>
<td>-</td>
<td>0.30 ± 0.06</td>
<td>0.32 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>15¹-Hydroxy-lactone chlorophyll a</td>
<td>6.00 ± 0.75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13²-Hydroxy-chlorophyll a</td>
<td>7.38 ± 0.58</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>233.6 ± 28.78</td>
<td>-</td>
<td>-</td>
<td>3.47 ± 0.01</td>
</tr>
<tr>
<td>Chlorophyll a’</td>
<td>21.13 ± 4.39</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15¹-Hydroxy-lactone-pheophytin a</td>
<td>13.31 ± 1.53</td>
<td>1.73 ± 0.38</td>
<td>1.70 ± 0.32</td>
<td>23.82 ± 0.67</td>
</tr>
<tr>
<td>15¹-Hydroxy-lactone-pheophytin a’</td>
<td>-</td>
<td>0.32 ± 0.04</td>
<td>0.30 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>13²-Hydroxy-pheophytin a</td>
<td>16.42 ± 1.87</td>
<td>0.77 ± 0.13</td>
<td>0.79 ± 0.06</td>
<td>26.01 ± 1.75</td>
</tr>
<tr>
<td>13²-Hydroxy-pheophytin a’</td>
<td>15.08 ± 2.18</td>
<td>0.72 ± 0.08</td>
<td>0.74 ± 0.07</td>
<td>31.22 ± 2.35</td>
</tr>
<tr>
<td>Pheophytin a</td>
<td>681.87 ± 99.63</td>
<td>3.82 ± 2.11</td>
<td>3.46 ± 0.84</td>
<td>478.11 ± 26.87</td>
</tr>
<tr>
<td>Pheophytin a’</td>
<td>136.41 ± 23.60</td>
<td>1.18 ± 0.46</td>
<td>1.08 ± 0.18</td>
<td>270.89 ± 14.21</td>
</tr>
<tr>
<td>Pyropheophytin a</td>
<td>210.58 ± 16.2</td>
<td>5.76 ± 3.17</td>
<td>4.32 ± 2.10</td>
<td>561.16 ± 36.28</td>
</tr>
<tr>
<td><strong>Total Chlorophylls</strong></td>
<td><strong>1448.4 ± 187.58</strong></td>
<td><strong>20.24 ± 9.19</strong></td>
<td><strong>19.35 ± 6.67</strong></td>
<td><strong>1520.70 ± 102.64</strong></td>
</tr>
</tbody>
</table>

| Chlorophylls (%) | 18.98 ± 1.19 | - | - | 0.22 ± 0.01 |
| Pheophorbides (%) | 6.88 ± 0.11  | 27.86 ± 2.73 | 34.36 ± 4.57 | 8.24 ± 0.25 |
| Pheophytins (%)   | 74.12 ± 2.62 | 70.65 ± 5.54 | 64.03 ± 5.30 | 90.99 ± 4.23 |
| Chlorin (%)       | -            | 1.48 ± 0.17  | 1.65 ± 0.07  | -     |
| Pyro-derivates (%)| 17.30 ± 0.60 | 42.68 ± 7.38 | 39.37 ± 5.56 | 42.61 ± 2.13 |
| Allomerized derivatives (%) | 4.23 ± 0.06 | 21.73 ± 0.72 | 20.36 ± 0.83 | 5.94 ± 0.27 |
Table 2: Effects of the inhibitor BLT1 on the rate of pheophorbide $a$ absorption by differentiated Caco-2 monolayers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absorption rate (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.16±0.68</td>
</tr>
<tr>
<td>BLT1 (μM)</td>
<td>Absorption (%)</td>
</tr>
<tr>
<td>0.1</td>
<td>56.4±5.02**</td>
</tr>
<tr>
<td>1</td>
<td>74.43±3.64*</td>
</tr>
<tr>
<td>10</td>
<td>72.66±3.67*</td>
</tr>
</tbody>
</table>

Experiments without treatment of inhibitor were considered as control (100%). Results are means ± S.E.M for three independent experiments. * denotes a significant difference from control, $p<0.05$ and ** $p<0.01$. 