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HPLC-hrTOF-MS study of copper chlorophylls: composition of food colorants and biochemistry after ingestion

Antonio PÉREZ-GÁLVEZ[1], Isabel VIERA[1], Itziar BENITO[2], María ROCA[1]*

[1]Food Phytochemistry Department, Instituto de la Grasa (CSIC), Campus Universitario, Building 46, 41013, Sevilla, Spain
[2]Laboratory Animal Services, University Hospital Virgen Macarena (HUVM), E-41009, Sevilla, Spain
*Corresponding author.
Email addresses: aperez@ig.csic.es (A. Pérez-Gálvez), iviera@ig.csic.es (I. Viera), ibenito@us.es (I. Benito), mroca@ig.csic.es (M. Roca).

Abstract

Despite the daily consumption of copper chlorophylls (E-141i), the green food colorants in foods high in fats, there is a general need for knowledge regarding their exact composition. Consequently, we have analyzed by HPLC-ESI(+)/APCI(+)-*hr*TOF-MS^2 the accurate composition of different commercial copper chlorophyll colorants for the first time. Data showed a favored yield of copper pheophytins from *a* series, while pheophytins from *b* series are preferentially no complexed with copper. The copper pheophytins present in the food colorants consisted mainly of three structural rearrangements. New fragmentation patterns and structural assignments have been described for several copper pheophytins. During the ingestion of copper chlorophylls, no chlorophyll derivative was present in serum nor urine except a new copper-pyroporphyrin *a* accumulated in a few livers. In any case, this green additive could represent the ideal food colorant, as most of the copper pheophytins are excreted in the feces showing almost no absorption of copper-chlorophylls compounds.

Keywords: composition, copper chlorophylls, copper pheophytins, E141i, food colorant, mass spectrometry.

Chemical compounds studied in this article

Pheophytin *a* (PubChem CID: 135398712); Pheophytin *b* (PubChem: 135407446); Pheophorbide *a* (PubChem CID: 253193); Copper Pheophytin *a* (PubChem: 6440851); Copper pheophytin *b* (PubChem: 135754270); 13^2-hydroxy-pheophytin *a* (PubChem: 136099746); Chlorin e_6 (PubChem CID: 5479494); Pyropheophorbide *a* (PubChem: 161456).

1. Introduction

Color is probably the main parameter that initially determines the choice of consumers and, consequently, the food industry reinforces the appearance of their products through allowed food colorants. In fact, the call of the consumers towards foods with a better appeal is empowering the food colorant industry. In this sense, the annual growth rate is projected to increase at 5.7%, to reach USD 5.12 billion by 2023 (Food colors markets, 2019). In addition, several food safety concerns are forcing the industry to replace synthetic food colors by natural colorants (Viera, Pérez-Gálvez & Roca, 2019). Among these, copper chlorophylls (E-141i) are the green natural food colorants authorized in the European Union (Regulation EC 1333/2008), Japan, China, and other countries for lipophilic foods. Currently, it is the responsible of the green coloration in ice-creams, cookies, dairy products, jellies, sweets, and several other food products (Martins, Lobo-Roriz, Morales, Barros & Ferreira, 2016) and consequently, it is produced and commercialized by the main food coloring companies. Copper chlorophyll products are obtained by the extraction of the pigment content of edible sources, alfalfa, lucerne or nettle, with specific organic solvents, a process which is followed by the addition of a copper salt to the extract. The insertion of copper stabilizes the chlorophyll compounds, ensuring a permanent green coloration during the rest of the processes applied for food manufacturing and during storage.

From a chemical perspective, the copper chlorophylls contained in the E-141i food colorants are a group of chlorophyll pigments with both the original porphyrin assembly and the phytyl ester chain of the native chlorophyll molecule but coordinated with a copper ion (Figure 1) (Roca, Gallardo-Guerrero, Mínguez-Mosquera & Gandul-Rojas, 2010). Hence, copper chlorophylls should be denoted as copper pheophytins (Commission Regulation (EU) No 231/2012), because the term chlorophyll is limited to those porphyrin structures coordinated with a magnesium ion. Additionally, some purity requirements have been included in the

legislation, mainly pointing to the allowed maximum levels of solvent residues and metals. Indeed, the use of copper chlorophylls derivatives is banned in some food products (Scotter, Castle & Roberts, 2005) and in some countries, or they are used in the food but without declaration in the food labeling. Initially, only chromatographic methods were developed to determine whether copper chlorophylls have been added or not to food. In the first studies, only the main components, copper chlorophyll *a* and *b* (actually copper pheophytins) were characterized (Inoue et al., 1988; Del Giovine & Fabietti, 2005; Scotter et al., 2005), remaining the majority of copper chlorophyll compounds unknown. However, the application of the expertise obtained with these pioneer chromatographic methodologies allowed the characterization of several commercial E-141i yields, concluding that they present a heterogeneous chlorophyll profile, where the main compound present in the samples is copper pyropheophytin instead of copper pheophytin (Roca et al., 2010, Figure 1). The prefix *pyro* means the lack of the carboxymethyl group at $C13^2$ (Figure 1, H in R_4). But the mentioned characterization was still based exclusively on chromatographic parameters. Since then, more accurate methods have been developed using HPLC-HRMS (Pérez-Gálvez, Ríos & Roca, 2015; Arrizabalaga-Larrañaga, Rodríguez, Medina, Santos & Moyano, 2019), or surface-enhanced Raman spectroscopy (Lian et al., 2015). But, those methods were able to detect exclusively copper pyropheophytin *a* or copper pheophytin for tracking the presence of E-141i colorant. The lack of authentic standards and the fact that most of the substituents in the chlorophyll tetrapyrrole does not modify the UV-Vis spectrum, difficult the proper identification of chlorophyll derivatives exclusively by its chromatographic properties, or even misidentifying the chlorophyll compounds. To the best of our knowledge, no precise identification of the whole array of copper chlorophyll compounds has been developed in commercial samples of E-141i by HPLC-MS. In this sense, the European food safety authority has published the need for accurate identification of the various compounds that are present in several food additives,

including in these requirements to the green food colorant E-141i (EFSA, 2015). In consequence, the European Commission has launched a call for scientific and technical data to complete the risk assessment, before taking the final decision on the status of the revised colorants (EC re-evaluation food additives program).

This lack of awareness causes uncertainty in the predictive capacity of both the chemical fate of the colorant in foods (each copper chlorophyll derivative might behave differently) and the performance of each compound during absorption and possible systemic distribution. Indeed, the potential bioactive effects attributed to chlorophylls, which have been recently reviewed (Pérez-Gálvez, Viera & Roca, 2017), should be reconsidered according to the actual characteristics of the chlorophyll profile, once the colorant has been added to the food, and after the digestive process has taken place. In this sense, several *in vitro* (Ferruzzi, Böhm, Courtney & Schwartz, 2002) and *in vivo* (Egner et al., 2000) assays have evaluated the availability of the polar green food colorant, E-141ii (copper chlorophyllins). However, despite the daily consumption of the green food colorant E-141i, no data are available on the fate of this colorant once assimilated.

According to these issues, there is a general claim from consumers, safety agencies, commercial suppliers and policymakers to address several questions including the complete identification of chlorophyll derivatives that shape the pigment profile of the green colorant and the acquisition of data regarding the fate of the E-141i once assimilated. Consequently, the hypothesis of the present study was to deal with the characterization of the colorant E-141i as a food additive, from the origin (commercial samples) to the end (animal excretion). With such aim, the composition of different non-polar green food colorants has been studied and subsequent analysis of the fate and metabolism of copper-chlorophylls once assimilated.

2. Materials and methods

2.1. Chemicals and raw material

Tetrabutylammonium acetate and ammonium acetate (purity >98%) were provided by Sigma-Aldrich (St. Louis, MO, USA). Solvents for LC were purchased from Teknokroma (Barcelona, Spain), while LC/MS grade solvents were supplied by Panreac (Barcelona, Spain). The deionized water was obtained from a Milli-Q 50 system (Millipore Milford, MA, USA). Chlorophyll *a* and *b* were purchased from Wako (Neuss, Germany) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The non-coppered chlorophylls (15^1-hydroxy-lactone-pheophytin *a*, 13^2-hydroxy-pheophytin *b*, 13^2-hydroxy-pheophytin *a* and pyropheophytin *b*) were produced from chlorophyll *a* and *b* in our laboratory with already published procedures (Chen, Ríos, Pérez-Gálvez & Roca, 202015a; Chen, Ríos, Roca & Pérez-Gálvez, 2015b). For copper chlorophylls, the corresponding non-coppered counterparts were dissolved in acetone for the chelation reaction with an excess of copper (II) ions as chloride and with ascorbic acid to reduce oxidative changes (Jones, White, Gibbs, Dennard & Agrie, 1968). The different samples of E-141i colorant (copper chlorophylls) were provided by diverse commercial suppliers of natural food colorants.

2.2. Experimental animals and diet

2.2.1. Diet preparation

Rodent-pellets (Harland Teklad) were supplied by ssniff-Spezialdiäten (Soest, Germany). Ground pellets were mixed with the selected commercial E-141i colorant (2% w/w) and water was added to improve homogenization and to obtain the original pellet-shape of the feed. The supplemented feed was dried in an oven at 30 ºC for 24 h to give consistency to the feed and avoid spoilage during storage. Supplemented feed was vacuum packed and stored at -20 ºC.

2.2.2. Mice experiment

24 C57BL/6N mice were obtained from the Animal Production and Experimentation Service (University of Seville, Spain). Three months old mice were housed in groups of 3-4 animals per cage upon arrival and were fed with the standard diet and water *ad libitum* for 14

days. Afterward, the mice were randomly separated into two groups. The control group (n = 8) received the standard diet, while the other group (n = 16) received the copper chlorophyll supplemented diet. Serum, urine and feces samples were collected weekly from both groups. At weeks 3 and 6, 3 mice of the control group and 6 mice of the copper chlorophyll supplemented diet group were euthanized by cervical dislocation and the livers were excised, weighed, rinsed with 0.9% saline solution and then stored at -80 ºC. The remaining 4 mice of the copper chlorophyll supplemented diet were fed with the standard diet for one additional week and subsequently, samples of feces were collected from both groups. The use of animals for this study and the procedures were carried out according to the Directive of the European Union (2010/63/EU) for the use of laboratory animals. This research was approved by the local Ethical Committee (study number 0158-N-15, Junta de Andalucía, Spain).

2.3. Extraction of chlorophyll derivatives from colorants, pellet, liver and feces samples

All procedures were performed under dimmed green light to avoid any photo-oxidation of chlorophylls. Livers, feces, and pellets were homogenized with 25 mL of acetone and then vacuum filtered. The operation was repeated until the solid filtrate was colorless. Subsequently, the extract was concentrated at 30 ºC in a rotary vacuum evaporator to dryness, and the dry residue was re-dissolved in 1 mL or 2 mL of acetone, for liver and feces extracts, or the pellet extract, respectively. Aliquots of commercial samples of copper chlorophyll colorants were dissolved in acetone. The extracts were filtered through a 0.22 μm nylon filter and stored at -20 ºC until chromatographic analysis, which was performed within 1 week.

2.4. Extraction of chlorophyll derivatives from serum and urine samples

Serum was isolated from blood samples by centrifugation at 3500 × g for 5 min and then an aliquot (0.15 mL) was mixed with 0.4 mL H_2O and 0.6 mL chloroform: methanol (1:1). The mixture was vortexed (30 s) and centrifuged (1500 × g for 5 min at 4 ºC). The chloroform layer was isolated, and the upper phase re-extracted again. The combined chloroform layers were

concentrated at 30 ºC in a rotary vacuum evaporator to dryness, and the dry residue was re-dissolved in 0.3 mL acetone, filtered through a 0.22 μm nylon filter and stored at -20 ºC until chromatographic analysis, which was performed within 1 week. The same extraction procedure was applied to the urine samples.

2.5. Identification and quantification of chlorophyll derivatives by HPLC–ESI(+)/APCI(+)-hrTOF–MS^n

The chromatographic analysis of the individual pigments (chlorophyll derivatives and carotenoids) was achieved in a Dionex Ultimate 3000RS U-HPLC equipment (Thermo Fisher Scientific, Waltham, MA, USA). We used a reversed-phase C18 column (Mediterranea Sea 18, 200×4.6 mm i.d., 3 μm particle size, Teknokroma, Barcelona, Spain), with the same elution gradient described previously (Chen et al., 2015a). The injection volume was 30 μL and the flow rate was set at 1 mL/min. The UV-Vis spectra of the chromatographic peaks were recorded with a PDA detector in the 300-700 nm range. The mass spectrometry instrument was a micrOTOF-QII^{TM} High-Resolution Time-of-Flight mass spectrometer (UHR-qTOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany). A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer ion source, while the analysis was developed with an APCI source (for phytylated chlorophyll derivatives) or an ESI interface (for dephytylated chlorophyll derivatives). The instrument was operated in positive ion mode and scanning the *m/z* values in the 50-1200 Da range, while the acquisition of the mass spectra was performed in broad-band Collision Induced Dissociation (bbCID) mode. Thus, MS and tandem MS spectra were recorded simultaneously. The instrument control was performed with Bruker Compass HyStar software (Bruker Daltonics version 3.2), whereas the processing of MS data was made with the Bruker Compass DataAnalysis software (Bruker Daltonics version 4.1). To allow an automatic screening of the signals corresponding to chlorophyll derivatives and carotenoids, we built an *in-house* database with the chromatographic behavior, UV-Vis features, and

theoretical monoisotopic exact mass values and isotopic patterns for each target (chlorophylls or carotenoids) compound. Then, we applied the TargetAnalysis™ software (Bruker Daltonics version 1.2) to the automatically extracted ion chromatograms (EICs) to allocate those EICs that tentatively match with the compounds included in the database. The validation of the automated tentative identification was carried out according to different filtering rules, including mass accuracy and isotopic pattern comparison calculated with the SigmaFit™ algorithm (tolerance limits set at 5 ppm and 50, respectively) (Chen et al., 2015a). The interpretation of the tandem MS spectra was performed considering that the consistency of the product ions must fulfil the previous filtering rules for mass accuracy and isotopic pattern. This procedure was developed with the SmartFormula3D™ module (Chen et al., 2015a). For those EICs that did not match with any compound included in the database, we applied a set of orthogonal criteria including chromatographic behavior, UV-Vis features, prediction of elemental composition with the SmartFormula3D™ module and application of the same filtering rules for the mass error and isotopic pattern of the corresponding EIC. Finally, the software MassFrontier™ (Thermo Scientific™ version 4.0, Waltham, USA) allowed the acquisition of the *in silico* tandem MS spectra for the unknown product, which was compared with the experimental product ions acquired in the bbCID mode.

Quantification was performed by PDA with the corresponding calibration curves (amount versus integrated peak area) of the identified pigments. For chlorin p_6 and phytyl-chlorin p_6, whose standards were not available, the calibration curve for pheophytin *a* was applied. The concentration ranges to build the equations were within the usual amounts of the pigments in the samples. Least-squares linear regression analysis was applied to the data to obtain the calibration curves. Triplicate analysis was made for five different volumes of each standard solution.

2.7. Experimental design and statistical analysis

The supplemented diet provided to mice is the single origin of copper chlorophylls, so the control group was fed with the standard rodent-diet free of any trace of chlorophyll derivatives. The mice that were fed with the supplemented diet did not change any 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 principles of the 3Rs and according to the guidelines and data from previous studies (Festing & Altman, 2002; Viera et al., 2018). 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 a post hoc comparison test. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Characterization by HPLC–ESI(+)/APCI(+)-hrTOF–MS^n of the chlorophyll derivatives present in E-141i green food colorants

The lipophilic version of the green food colorants (E-141i), has received less attention than the hydrophilic counterpart (E-141ii or copper chlorophyllins) (Roca et al., 2010; Fang et al., 2015). Figure 2 depicts the HPLC chromatograms of three commercial E-141i green food colorants. The current legislation restrains the use of edible green plants as the unique raw material for the production of the E-141i. Consequently, the different pigment profiles observed in Figure 2 are a consequence of the diverse plant materials used and/or the different processing methods. Indeed, the main chlorophyll derivative in the E-141i green food colorants is the copper-pyropheophytin *a* (peak 18, in Figure 2), as it has been denoted previously either by HPLC-UV-Vis means (Del Giovine & Fabietti, 2005; Scotter et al., 2005; Roca et al., 2010) or with the application of HPLC-MS (Fang et al., 2015; Pérez-Gálvez et al., 2015; Arrizabalaga-Larrañaga et al., 2019). However, the focus of this study was to deal with the rest of the

chlorophyll derivatives also present in the E-141i yields. Hence, 13 different chlorophyll derivatives have been identified in the colorants, which chromatographic and MS characteristics are outlined in Table 1. All the chlorophyll compounds have been identified based on their spectroscopic features, exact mass, elemental composition, isotopic pattern and when possible co-injection with authentic standards. Some of them have been previously characterized in other sources (plants or foods), while others are characterized by the first time in this study. As it was anticipated, most of the main chlorophyll pigments in the green lipophilic colorants are chelated with copper, while only a small number of chlorophyll derivatives presents hydrogen atoms at the center of the macrocycle (peaks 7, 9, 11 and 14). The parent chlorophyll *a* and *b* structures comprised of the original green raw materials commonly used for the production of E-141i (alfalfa, lucerne or nettle) contain Mg^{+2} coordinated in the macrocycle. However, this metal is easily replaced by hydrogen atoms in light acidic conditions or during storage, which are easily prevailed during the processing of the green tissues for production of E-141i. The following treatment with copper salts induces the formation of copper chlorophyll derivatives as copper is easily accomplished in the central macrocycle (Petrović, Nikolić & Marković, 2006) through a highly favorable reaction (Bechaieb, Fredj, Akacha & Gerard, 2016). In fact, the insertion of copper into the chlorophyll molecule stabilizes the structure (Viera et al., 2019). Another common feature in the analyzed E-141i colorants is the predominant occurrence (except the peak 5) of pheophytins, that is chlorophyll derivatives that present a phytyl chain at $C17^3$, (Figure 1), a feature that preserves the lipophilic character of the E-141i colorants (Inoue et al., 1988; Scotter et al., 2005; Roca et al., 2010). Additionally, the relative presence of chlorophyll derivatives from *a* series (featured by a CH_3 at C7, Figure 1) and *b* series (featured by a CHO residue at C7, Figure 1) denotes the ratio of both series in the raw material. In any case, it is noteworthy to account that the main representative pigment of *b* series in all the analyzed E-141i colorants is not a copper derivative,

while 90-100% of chlorophyll derivatives of *a* series are copper complexes. This result is in agreement with previous studies that showed the faster production of copper complexes of *a* series than that of *b* series (Jones, White, Gibbs, Butler & Nelson, 1977). Independently of the chlorophyll series, further structural rearrangements observed in the pheophytin structure in the E-141i colorants are oxidation at the $C13^2$ with the subsequent formation of a lactone group at $C15^2$, and the loss of the carboxymethyl group at $C13^2$ yielding the corresponding pyroderivatives (Figure 1). All these arrangements are common structures arising from the natural metabolism of chlorophylls, food processing techniques and even food storage (Schwartz & Lorenzo, 1990).

Notwithstanding, as stated before, only the main copper chlorophyll in the food colorants, copper pyropheophytin *a* (peak 18) has been characterized in detail (Zvezdanović, Petrović, Marković, Andjelković & Andjelković, 2014; Roca et al., 2015). Also, several product ions of copper pheophytin *a* and *b* (peaks 10, 15 and 17) and copper pyropheophytin *b* (peak 16) (Aparicio-Ruiz, Riedl & Schwartz, 2011) have been described. However, special attention should be noted for the correct identification of the parent ions to avoid misidentifications. Among other standards, the consistency of the isotopic pattern between the product ions and the corresponding parent ion should be preserved to assure the origin of the product ions. To fulfil this standard, all the product ions included in Table 1 have been filtered with the isotopic pattern criterion (mSigma below 50, see section 2.5). Indeed, the application of this standard allowed the identification of new product ions, which are proposed in this study to fully characterize the above-mentioned copper chlorophylls, and new structural assignments have been determined for those product ions with the assistance of Mass Frontier™ software. Only a few studies consider detailed fragmentation patterns (Zvezdanović et al., 2014). In this sense, the mass spectrometry behavior of non-copper chlorophyll derivatives (peaks 7, 9, 11 and 14) is already known (Viera, Roca & Pérez-Gálvez, 2018), with fragmentation patterns associated

285 to each structural configuration. However, to the best of our knowledge, several of the
286 chlorophyll compounds identified in the copper chlorophyll food colorants have not been
287 previously characterized. For example, the UV-Vis features of copper pheophorbide *a* and *b*
288 (peaks 3 and 4) were established more than 50 years ago (Jones et al., 1968), but we did not
289 find any study related with their MS behavior, despite their presence in table olives (Aparicio-
290 Ruiz et al., 2011) and the E-141i colorant (Roca et al., 2010). Starting with peaks 2 and 3 (Table
291 1) and under our experimental conditions, the main product ion for both pheophorbides
292 corresponds with the fragmentation of the $C_2H_4O_2$ group along the propionic chain at C17.
293 Curiously, the same fragmentation pattern was observed for the corresponding non-copper
294 counterparts, pheophorbide *a* and *b* (Van Breemen et al., Canjura & Schwartz, 1999; Chen et
295 al., 2015b). In the same line, copper pyropheophorbide *a* (peak 5) has been identified in both
296 polar (Mortensen & Geppel, 2007) and apolar (Roca et al., 2010) green food colorants, but it
297 has not been the focus of MS studies. We found as the main product ion, the fragmentation of
298 a CO group (Table 1). Mass Frontier^{TM} assigned such fragmentation for copper
299 pyropheophorbide *a* taking place at the isocyclic ring (ring V, Figure 1). On the contrary, the
300 products ions of pyropheophorbide *a* are limited to the MS^2-reactions involving the propionic
301 acid group at C17. Interestingly, the CO losses are related exclusively with *b* series for non-
302 copper chlorophylls (Viera et al., 2018b). For this structural configuration, the insertion of
303 copper significantly modifies the MS fragmentation pattern. The new main MS^2 fragmentations
304 found for the peaks 10, 12, 13, 15, 16 and 17 (Table 1), as phytylated chlorophylls, are
305 associated with losses of the phytyl chain (Viera et al., 2018b) along with further
306 fragmentations. Among them, peak 12 (copper-13^2-hydroxy-pheophytin *a*) and peak 13
307 (copper-15^1-hydroxy-lactone-pheophytin *a*) are common pigments in all the food colorants
308 analyzed (Figure 2). The main product ion of the 13^2-hydroxy derivative of copper pheophytin
309 *a* (peak 12) also implies the fragmentation of a CO group. However, for the non-copper

hydroxy-chlorophylls, the typical MS product ions included the hydroxyl group at $C13^2$ (Viera et al., 2018b). On the other hand, the main product ion of copper-15^1-hydroxy-lactone-pheophytin *a* (peak 13, Table 1) was assigned to the loss of phytadiene plus the opening of the isocyclic ring ($C_3H_5NO_2$). The opening of the lactone function and subsequent rearrangements of the remaining groups at the isocyclic ring originate exclusive product ions in non-copper 15^1-hydroxy-lactone-chlorophylls (Viera et al., 2018b). For copper pheophytin *a* and its epimer (peaks 15 and 16), the new MS^2 fragment ions found can be assigned to sequential losses of the phytadiene unit besides an acetic acid group from the propionic chain at $C17^3$ (Figure 1). Those fragmentations are the same as in their non-metal counterparts (pheophytin *a*, Chen et al., 2015a). However, the MS^2 behavior of copper pyropheophytin *b* (peak 16) is completely different from its non-copper relative (pyropheophytin *b*, Chen et al., 2015a). Its main product ion (*m/z* 582.1325) can be assigned, besides the characteristic phytadiene unit, to an unexpected ethylene fragmentation. At present, the influence of the copper insertion in the chlorophyll macrocycle in the MS behavior is scarcely understood, as only pioneer studies have been developed (Zvezdanović et al., 2014). Our data suggest a distinct behavior depending on the structural configuration of the copper chlorophyll derivative. In any case, the main product ions described for the different chlorophyll derivatives present in the copper-chlorophyll E-141i food colorants are a stronger demanding parameter to control the presence and quality of those green natural colorants.

3.2. Biochemistry of the green food colorant E-141i after ingestion.

To mimic the performance of the lipophilic green food colorants once ingested, experimental mice were fed with E-141i-rich and control pellets for six weeks. Copper chlorophyll pellets were produced with the heterogeneous and more complex E-141i colorant (corresponding with one shown in Figure 2C). Remarkably, the E-141i-pellet (Figure 3A) contains some minor compounds that were not present in the original E-141i green colorant,

335 which were identified according to their UV-Vis spectra and tandem MS features. Hence,
336 hydroxy-copper-pheophorbide derivative and both phytylated and dephytylated chlorin p_6
337 derivatives appeared (peaks 1, 2 and 19 in Figure 2), and they represent *ca*. less than 2% of the
338 total chlorophyll content in the supplemented diet. The hydroxylation and the opening of the
339 isocyclic ring (ring V, Figure 1) should be considered as common transformation products
340 arising from the parent chlorophyll structure, which is prone to changes already denoted during
341 the thermal process (Schwartz et al., 1990). Chlorin p_6 and its phytylated counterpart belong to
342 the group of chlorophylls characterized by an open isocyclic ring, and particularly with two
343 carboxylic acids at C13 and C15 (Figure 1). Chlorin p_6 is the substrate for the development of
344 potent photosensitizers for photodynamic therapy (Meng et al., 2016) while copper chlorin p_6
345 is a common chlorophyll derivative present in the polar colorant E-141ii (copper
346 chlorophyllins) (Mortensen et al., 2007), but there are no references of mass spectrometry
347 studies. Anyway, the exact mass, the elemental composition, the retention time and the UV-Vis
348 spectrum of peaks 1 and 19, allow us to identify them as chlorin p_6 and its corresponding
349 phytylated derivative. Unfortunately, it was impossible to develop the MS^2 study with chlorin
350 p_6 due to the low presence of this chlorophyll derivative in the enriched pellets, but it was
351 possible to perform such study with the phytyl-chlorin p_6. The main product ion of this open-
352 ring chlorophyll derivative is a m/z = 585.2710 (Table 1). Such fragmentation (with the
353 assistance of Mass Frontier^{TM}) was assigned to the loss of a phytadiene unit, which is related to
354 the behavior observed for other phytyl-chlorophylls. Although deep studies are required, it
355 seems the opening of the isocyclic ring does not greatly influence the mass spectrometry
356 behavior. Peak 2 is tentatively identified as copper-13^2-hydroxy-pheophorbide *a*, considering
357 the exact mass, the elemental composition and retention time. The recorded UV-Vis for this
358 peak with maxima at 400, 425 and 650 nm is similar to the copper pheophorbide *a* (Table 1) as
359 expected because the hydroxylation at C13^2 does not modify the absorption features, although

no previous characterization has been found. Its MS^2 analysis displayed the product at *m/z* = 520.0595 as the main product ion. The Mass Frontier™ software assigned the corresponding product ions with losses along with the propionic acid group at C17 and further ring opening, an additional step with respect to its parent copper-pheophorbide *a* (section 3.1). However, the main product ion is not the loss of the hydroxyl group at $C13^2$ as it occurs with the non-coppered hydroxylated chlorophylls (Viera et al., 2018b). As stated earlier, the insertion of copper ions modifies or not the MS behaviors of chlorophyll compounds compared with non-chelated chlorophylls (Zvedonzić et al., 2014) depending on the structural configuration. However, further studies are required to establish the general fragmentation patterns of copper chlorophylls. Anyhow, besides these three minor new chlorophyll derivatives, the rest of chlorophylls in the enriched pellet were the same as the colorant (Fig 2C and 3A).

To the best of our knowledge, there is no data available on the behavior of the food colorant E-141i once assimilated. In this regard, the analysis of the extracts isolated from serum (Figure 3B) and urine (Figure 3C) samples from the mice group fed with the E-141i-supplemented diet revealed the absence of chlorophyll derivatives during the 6 weeks of the assay. These data pointed out to the almost absence of intestinal absorption of the chlorophyll derivatives present in the natural colorant E-141i, as none of them reach the circulatory system. A similar trend can be observed in the liver samples (Figure 4A), except for one copper-chlorophyll derivative that was observed at trace level in some of the liver samples from the E-141i-supplemented diet mice group, at week 3 and 6. The characterization of the compound was based on two criteria relative to accurate mass and tandem MS, isotopic pattern, features of the UV-Vis spectrum and chromatographic behavior. The compound elutes at 4.8 min in the region of the dephytylated chlorophyll derivatives and the UV-Vis spectrum shows one main maximum located at 415 nm and two small peaks at 545 and 582 nm (Figure 4B). The lack of a second maximum in the red absorption spectra (630-660 nm) is characteristic of porphyrins

(Mortensen & Geppel, 2007), which are chlorophylls with a double-bound between C17 and C18 (Sheer, 2006). The protonated molecular ion was observed at *m/z* = 598.1630 with a characteristic isotopic profile of the copper-derivatives where the intensity of the signal corresponding to the third isotope (*m/z* = 600.1640) is higher than the observed for the second isotope (*m/z* = 599.1632), a consequence of the contribution of copper to the isotopic profile of the molecule (Mortensen & Geppel, 2007). This characteristic is useful for the localization of metallo-chlorophyll derivatives, i.e. copper- and zinc-chlorophylls (Van Breemen et al., 1991) in HPLC-MS analysis of samples containing chlorophyll products. The application of algorithms for the prediction of the elemental composition considering the mass error and mSigma filters (see section 2.5) yielded a molecular formula for the protonated ion equal to $C_{32}H_{30}CuN_4O_4$ (mass error = 2.9 ppm, mSigma value = 28). Two product ions (Figure 4C) were also observed at *m/z* = 556.1532 and at *m/z* = 512.1271. The former product ion (peak 2 in Figure 4C) with an elemental composition $C_{30}H_{30}CuN_4O_3$ corresponds with the fragmentation at the isocyclic ring and involving the carbon atoms at 13^1 and 13^2 positions. The second product ion (peak 3 in Figure 4C), with an elemental composition $C_{30}H_{30}CuN_4O_3$, is produced after the loss of the propionic chain at C17, a characteristic process during the acquisition of tandem MS spectra of chlorophyll derivatives (Viera et al., 2018b). With this information, we tentatively identify this product as a copper pyroporphyrin *a*, with a structure depicted in Figure 4C. The proposed structure exhibits a free propionic acid at C17, characteristic of most porphyrins (Scheer, 2006), which confers high polarity, a hydroxyl group at C3 and a lack of the carboxymethyl group at $C15^2$. However, the presence of this compound in the liver should not be odd. The liver is considered the organ where the metabolism (biosynthesis and partially catabolism) of heme compounds, which are also porphyrins, takes place lacking the carboxymethyl group at $C15^2$. Consequently, the enzymatic systems, transporters, etcetera, from the liver would recognize this chlorophyll derivative as a common structure. We can

hypothesize that the portal system could be the mechanism of absorption from the intestine to the liver and that the set of copper-pheophorbide derivatives in the pellets should be considered as the main source of that metabolite product. We already demonstrated that the more polar chlorophylls (pheophorbide and pyropheophorbide) are preferentially stored in the liver of C57BL/6N mice fed with a spirulina-supplemented diet (Viera et al., 2018a). This favored accumulation was attributed in part to the selectivity of the SR-BI lipid transported towards pheophorbide derivatives.

On the contrary, the feces (Figure 3D) contained the same chlorophyll derivative profile observed in the supplemented diet (Figure 3A), except for the most polar chlorophyll compounds, chlorin p_6 and several copper- pheophorbides (peaks 1 to 4) which were present in the E-141-pellets but not in the feces at any sampling period. To quantitatively compare the chlorophyll content of the pellet and the feces, we quantified all the chlorophyll derivatives during the six weeks of the assay. Data on Table 2 shows that quantitatively the chlorophyll compounds in pellet and feces are statistically equal. Although occasionally in one week, there could be a significant difference ($P < 0.05$) for a specific chlorophyll compound between pellets and feces, such divergence was non-consistent along the six weeks of study, being consequently attributed to the individual variability. These data indicate that almost no chlorophyll compound from E-141i colorants is transformed during the digestion process and that the copper chlorophyll absorption is imperceptible. Previous studies, which were performed both *in vitro* and *in vivo* (Ferruzzi, Failla & Schwartz, 2001; Chen & Roca, 2018), showed the different reactions that take place over the natural chlorophylls (Mg-derivatives) during the digestion, but none of them occurs with the chlorophyll compounds present in the copper chlorophyll colorants (Table 2). Thus, *in vivo* absorption studies of non-coppered chlorophylls showed a selective absorption of chlorophyll derivatives (Viera et al., 2018a). However, data presented in Table 2 demonstrate that almost imperceptible absorption of chlorophylls present in copper

chlorophyll colorant takes place. Even more, once the E-141i supplemented diet was suspended at week 6, we analyzed the feces samples one week later, and no chlorophyll derivatives were detected in the extracts, and the serum, urine and liver samples continued free of chlorophyll derivatives and metabolites.

4. Conclusion

Copper chlorophylls colorants (E-141i) are an excellent example of how a straightforward modification (coordination with copper) of the natural chlorophyll (Mg-derivatives and pheophytins) produces an outstanding result in terms of stability of the final coloring stuff aimed to use in foods. However, the characteristics and attributes observed for the natural chlorophyll derivatives should not be directly extended to the copper chlorophylls, which could be considered nature-identical products. Our study showed the exact composition of the copper-chlorophylls that shape the pigment profile of the E-141i colorants, with special emphasis on the mass spectroscopic behavior. Commercial samples of the green food colorant E-141i are mainly composed of copper pheophytins, although the proportion is different for *a* series (almost 100%) than for *b* series (with most of the non-copper pheophytins). Three main structural rearrangements are observed in the different pheophytin structures present in the E-141i colorants: oxidation at the $C13^2$ with the subsequent formation of a lactone group at $C15^1$, and the loss of the carboxymethyl group at $C13^2$ yielding the corresponding pyroderivatives. The high resolving power of the methodology used assisted by robust post-processing software for the management of data has allowed characterizing by the first time the fragmentation patterns and structural assignments for several copper chlorophyll compounds (copper pheophorbide *a*, copper pheophorbide *b*, copper pyropheophorbide *a*, copper 13^2-hydroxy-pheophytin *a*, and copper 15^1-hydroxy-lactone-pheophytin *a*). In addition, new (and alternative) main product ions have been proposed for other copper pheophytins present in the corresponding green food colorants. Finally, according to our results, it can be concluded that

460 the food colorant E-141i could be considered the "ideal food colorant" as it is almost completely
461 excreted with a very faint absorption, which minimizes the risk of interaction with the body.

462 **Author contributions**

463 Antonio Pérez-Gálvez: conceptualization, data curation, investigation, software and
464 review writing; Isabel Viera: formal analysis, investigation, methodology and original draft
465 writing; Itziar Benito: conceptualization, investigation and draft writing; María Roca:
466 conceptualization, data curation, funding acquisition, Project administrator, supervision and
467 review/editing writing.

468 **Declaration of competing interest**

469 The authors declare no conflict of interest.

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478 **References**

479 Aparicio-Ruiz, R., Riedl, K.M., & Schwartz, S.J. (2011). Identification and Quantification of
480 Metallo–Chlorophyll Complexes in Bright Green Table Olives by High-Performance Liquid
481 Chromatrography–Mass Spectrometry Quadrupole/Time-of-Flight. *Journal of Agricultural*
482 *and Food Chemistry, 59*, 11100-11108. https://doi. 10.1021/jf201643s.

483 Arrizabalaga-Larrañaga, A., Rodríguez, P., Medina, M., Santos, F.J., & Moyano, E. (2019).
484 Simultaneous analysis of natural pigments and E-141i in olive oils by liquid chromatography-

tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *411*, 5577–5591. https://doi.org/10.1007/s00216-019-01939-3.

Bechaieb, R., Fredj, A.B., Akacha, A.B., & Gerard, H. (2016). Interactions of copper(II) and zinc(II) with chlorophyll: insights from density functional theory studies. *New Journal of Chemistry*, *40*(5), 4543-4549. https://doi.org/10.1039/C5NJ03244J.

Clarke, P.A, Barnes, K.A., Startin, J.R., Ibe, F.I., & Shepherd, M.J. (1996). High Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometry for the Determination of Carotenoids. *Rapid Communications in Mass Spectrometry 10*, 1781-1785. https://doi.org/10.1002/(SICI)1097-0231(199611)10:14<1781::AID-RCM748>3.0.CO;2-D.

Chen, K., Ríos, J.J., Pérez-Gálvez, A., & Roca, M. (2015a). Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (I) Phytylated derivatives. *Journal of Chromatography A, 1406*, 99-108. https://doi.org/10.1016/j.chroma.2015.05.072.

Chen, K., Ríos, J. J., Roca, M., & Pérez-Gálvez, A. (2015b). Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (II) Dephytylated derivatives. *Journal of Chromatography A, 1412*, 90-99. https://doi.org/10.1016/j.chroma.2015.08.007.

Chen, K., & Roca, M. (2018). In vitro digestion of chlorophyll pigments from edible seaweeds. *Journal of Functional Foods*, *40*, 400-407. https://doi.org/10.1016/j.jff.2017.11.030.

De Rosso, V.V., & Mercadante, A.Z. (2007). Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *Journal of the Agricultural Food Chemistry, 55*, 5062-5072. https://doi.org/10.1021/jf0705421.

508 Del Giovine, L., & Fabietti, F. (2005). Copper chlorophyll in olive oils: Identification and
509 determination by LIF capillary electrophoresis. *Food Control, 16,* 267–272.
510 https://doi.org/10.1016/j.foodcont.2004.03.005.
511 EC re-evaluation food additives programme. Food colorants: Challenges, opportunities and
512 current desires of agro-industries to ensure consumer expectations and regulatory practices.
513 https://ec.europa.eu/food/safety/food_improvement_agents/additives/re-evaluation_en/
514 Accessed 3 January 2020.
515 EFSA Panel on Food Additives and Nutrient Sources added to Food. (2015). Scientific Opinion
516 on the Re-Evaluation of Chlorophylls (E 141 (i and ii)) as Food Additives. EFSAJ. 2015, 13,
517 4151. https://www.efsa.europa.eu/en/efsajournal/pub/4151/ Accessed 2 January 2020.
518 Egner, P.A., Stansbury, K.H., Snyder, E.P., Rogers, M.E., Hintz, P.A., & Kensler, T.W. (2000).
519 Identification and characterization of chlorin e4 ethyl ester in sera of individuals participating
520 in the chlorophyllin chemoprevention trial. *Chemical Research in Toxicology*, *13*, 900-906.
521 https://doi.org/10.1021/tx000069k.
522 Egner, P. A., Wang, J.-B., Zhu, Y.-R., Zhang, B.-C., Wu, Y., Zhang, Q.-N., Qian, G.-S., Kuang,
523 S.-Y., Gange, S. J., Jacobson, L. P., Helzlsouer, K. J., Bailey, G. S., Groopman, J. D., &
524 Kensler, T. W. (2001). Chlorophyllin intervention reduces aflatoxin-DNA adducts in
525 individuals at high risk for liver cancer. *Proceedings of the National Academy of Sciences,*
526 *98*(25), 14601-14606. https://doi.org/10.1073/pnas.251536898.
527 Fang, M., Tsai, C.-F., Wu, G.-Y., Tseng, S.-H., Cheng, H.-F., Kuo, C.-H., Hsu, C.-L., Kao, Y.-
528 M., Shih, D. Y.-C., & Chiang, Y.-M. (2015). Identification and quantification of Cu-chlorophyll
529 adulteration of edible oils. *Food Additives & Contaminants: Part B, 8*(3), 157-162.
530 https://doi.org/10.1080/19393210.2015.1025861.
531 Ferruzzi, M. G., Bohm, V., Courtney, P. D., & Schwartz, S. J. (2002). Antioxidant and
532 Antimutagenic Activity of Dietary Chlorophyll Derivatives Determined by Radical Scavenging

533 and Bacterial Reverse *Mutagenesis Assays. Journal of Food Science*, *67*(7), 2589-2595.
534 https://doi.org/10.1111/j.1365-2621.2002.tb08782.x .
535 Ferruzzi, M. G., Failla, M. L., & Schwartz, S. J. (2001). Assessment of degradation and
536 intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in
537 vitro digestion and Caco-2 human cell model. *Journal of Agricultural and Food Chemistry*, *49*,
538 2082–2089. https://doi.org/10.1021/jf000775r.
539 Festing, M. F. W., & Altman, D. G. (2002). Guidelines for the Design and Statistical Analysis
540 of Experiments Using Laboratory Animals. *ILAR Journal*, *43*(4), 244-258.
541 https://doi.org/10.1093/ilar.43.4.244.
542 Food colors market. Markets and markets. Food Colors Market by Type (Natural, Synthetic,
543 Nature-Identical), Application (Beverages, Processed Food, Bakery & Confectionery Products,
544 Oils & Fats, Dairy Products, Meat, Poultry, Seafood), Form, Solubility, and Region - Global
545 Forecast to 2023 Available online: https://www.marketsandmarkets.com/Market-Reports/food-
546 colors-market-
547 36725323.html?gclid=EAIaIQobChMIpumD692H5gIV2YjVCh32GAqSEAAYASAAEgLeG
548 fD_BwE. (accessed on 2 January 2020).
549 Inoue, H., Furuya, K., Watanabe, K., Tanaka, K., Shirai, T., & Miyoshi, E. (1988). Separation
550 and determination of copper(II) chlorophylls by reversed-phase high performance liquid
551 chromatography. *Analytical Sciences*, *4*(6), 599-603. https://doi.org/10.2116/analsci.4.599.
552 Jones, I. D., White, R. C., Gibbs, E., Butler, L. S., & Nelson, L. A. (1977). Experimental
553 formation of zinc and copper complexes of chlorophyll derivatives in vegetable tissue by
554 thermal processing. *Journal of Agricultural and Food Chemistry*, *25*(1), 149-153.
555 https://doi.org/10.1021/jf60209a030.

556 Jones, I. Dunlavy., White, R. Cyrus., Gibbs, Eleanor., & Denard, C. D. (1968). Absorption
557 spectra of copper and zinc complexes of pheophytins and pheophorbides. *Journal of*
558 *Agricultural and Food Chemistry, 16*(1), 80-83. https://doi.org/10.1021/jf60155a001.
559 Lian, W., Shiuec, J., Wang, H., Hong, W., Shih, P.H., Hsu, C.K., Huang,C.Y., Hsing, C.R.,
560 Wei,C.M., Wang, J.K. & Wang, Y.L. (2015). Rapid detection of copper chlorophyll in
561 vegetable oils based on surface-enhanced raman spectroscopy. *Food Additives &*
562 *Contaminants, 32*, 627–634. https://doi.org/10.1080/19440049.2015.1014867.
563 Martins, N., Roriz, C. L., Morales, P., Barros, L., & Ferreira, I. C. F. R. (2016). Food colorants:
564 Challenges, opportunities and current desires of agro-industries to ensure consumer
565 expectations and regulatory practices. *Trends in Food Science & Technology, 52,* 1-15.
566 https://doi.org/10.1016/j.tifs.2016.03.009.
567 Meng, Z., Yu, B., Han, G., Liu, M., Shan, B., Dong, G., Miao, Z., Jia, N., Tan, Z., Li, B., Zhang,
568 W., Zhu, H., Sheng, C., & Yao, J. (2016). Chlorin p 6 -Based Water-Soluble Amino Acid
569 Derivatives as Potent Photosensitizers for Photodynamic Therapy. *Journal of Medicinal*
570 *Chemistry, 59*(10), 4999-5010. https://doi.org/10.1021/acs.jmedchem.6b00352.
571 Mortensen, A., & Geppel, A. (2007). HPLC–MS analysis of the green food colorant sodium
572 copper chlorophyllin. *Innovative Food Science & Emerging Technologies, 8*, 419-425.
573 https://doi.org/10.1016/j.ifset.2007.03.018.
574 Pérez-Gálvez, A., Ríos, J.J., & Roca, M. (2015). A new probe for tracking the presence of E141i
575 food colorant. *Food Control, 51*, 240–243. https://doi.org/10.1016/j.foodcont.2014.11.028
576 Perez-Galvez, A., Viera, I., & Roca, M. (2018). Chemistry in the Bioactivity of Chlorophylls:
577 An Overview. *Current Medicinal Chemistry, 24*(40).
578 https://doi.org/10.2174/0929867324666170714102619.

579 Petrovic, J., Nikolic, G., & Markovic, D. (2006). In vitro complexes of copper and zinc with
580 chlorophyll. *Journal of the Serbian Chemical Society, 71*(5), 501-512.
581 https://doi.org/10.2298/JSC0605501P.
582 Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December
583 2008 on Food Additives. https://eur-lex.europa.eu/eli/reg/2008/1333/2016-05-25/ Accessed 2
584 January 2020).
585 Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for
586 food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European
587 Parliament and of the Council Text with EEA relevance. https://eur-
588 lex.europa.eu/eli/reg/2008/1333/2016-05-25/ Accessed 2 January 2020.
589 Roca, M., Gallardo-Guerrero, L., Mínguez-Mosquera, M. I., & Gandul Rojas, B. (2010).
590 Control of Olive Oil Adulteration with Copper−Chlorophyll Derivatives. *Journal of*
591 *Agricultural and Food Chemistry, 58*(1), 51-56. https://doi.org/10.1021/jf902084d.
592 Scotter, M.J., Castle, L., & Roberts, D. (2005). Method development and HPLC analysis of
593 retail foods and beverages for copper chlorophyll (E141[i]) and chlorophyllin (E141[ii]) food
594 colouring materials. *Food Additives & Contaminants, 12*, 1163–1175. https://doi.
595 10.1080/02652030500306885.
596 Sheer, H. (2006). An Overview of Chlorophylls and Bacteriochlorophylls: Biochemistry,
597 Biophysics, Functions and Applications. In Grimm, B., Porra, R.J., Rüdiger, W., & Scheer, H.
598 (Eds.) *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and*
599 *Applications* (pp.1-26). Netherlands: Springer.
600 Schwartz, S.J., & Lorenzo, T.V. (1990). Chlorophylls in foods. *Critical Reviews in Food*
601 *Science and Nutrition, 29*, 1-17. https://doi.org/10.1080/10408399009527511.

602 Van Breemen, R.B., Canjura, F.L., & Schwartz, S.J. (1991). Identification of Chlorophyll
603 Derivatives by Mass Spectrometry. *Journal of Agricultural and Food Chemistry, 39*, 1452-
604 1456. https://doi.org/10.1021/jf00008a018.
605 Viera, I., Chen, K., Ríos, J.J., Benito, I., Pérez-Gálvez, A., & Roca, M. (2018a). First-pass
606 metabolism of chlorophylls in mice. *Molecular Nutrition and Food Research, 62*, 1800562.
607 https://doi.org/10.1002/mnfr.201800562.
608 Viera, I., Pérez-Gálvez, A., & Roca, M. (2018b). Mass spectrometry of non-allomerized
609 chlorophylls *a* and *b* derivatives from plants. *Current organic chemistry, 22*, 842-876.
610 https://doi.org/10.2174/1385272821666170920164836.
611 Viera, I., Pérez-Gálvez, A., & Roca, M. (2019). Green Natural Colorants. *Molecules, 24*(1),
612 154. .
613 Zvezdanović, J.B., Petrović,S.M., Marković,D.Z., Andjelković,T.D., & Andjelković, D.H.
614 (2014). Electrospray ionization mass spectrometry combined with ultrahigh performance liquid
615 chromatography in the analysis of in vitro formation of chlorophyll complexes with copper and
616 zinc. *Journal of the Serbian Chemical Society, 79*, 689–706. https://doi.
617 10.2298/JSC130918009Z.

618 **Figure captions**

619 Figure 1: Structural configuration names and numbering system for all the chlorophylls
620 derivatives identified by HPLC-ESI/APCI-hrTOF- MS/MS in the samples analyzed.

621 Figure 2. HPLC traces acquired at 430 nm corresponding to the analysis of the pigment profile
622 of three commercial E-141i colorants. Number for the identification corresponds to: 3, copper
623 pheophorbide *a*; 4, copper pheophorbide *b*; 5, copper pyropheophorbide *a*; 6, lutein; 6', *cis*-
624 lutein; 7, 15^1-hydroxy-lactone-pheophytin a; 8, β-cryptoxanthin; 9, 13^2-hydroxy-pheophytin *b*;
625 10, copper pheophytin *b*; 11, 13^2-hydroxy-pheophytin *b*; 12, copper 13^2-hydroxy-pheophytin

626 *a*; 13, copper 15^1-hydroxy-lactone-pheophytin *a*; 14, pyropheophytin *b*; 15, copper pheophytin
627 *a*; 16, copper pyropheophytin *b*; 17, copper pheophytin *a'*; 18, copper pyropheophytin *a*.
628 Figure 3. HPLC traces acquired at 430 nm corresponding to the analysis of the pigment profile
629 of the E-141i supplemented diet (A), serum (B), urine (C) and the feces (D) from mice that
630 were fed with E-141i-pellets (week 3). Number for the identification corresponds to: 1, chlorin
631 p_6; 2, copper 13^2-hydroxy-pheophorbide *a*; 3, copper pheophorbide *a*; 4, copper pheophorbide
632 *b*; 5, copper pyropheophorbide *a*; 6, lutein; 6', *cis*-lutein; 8, β-cryptoxanthin; 9, 13^2-hydroxy-
633 pheophytin b; 10, copper pheophytin *b*; 12, copper 13^2-hydroxy-pheophytin *a*; 13, copper 15^1-
634 hydroxy-lactone-pheophytin *a*; 14, pyropheophytin *b*; 15, copper pheophytin *a*; 16, copper
635 pyropheophytin *b*; 17, copper pheophytin *a'*; 18, copper pyropheophytin *a*; 19, phytyl-chlorin
636 p_6.
637 Figure 4. HPLC trace (A) acquired at 430 nm corresponding to the analysis of the pigment
638 profile in a liver extract from mice fed with the E-141i supplemented diet. UV-Vis spectrum
639 of the peak at 4.8 min (B) and MS spectrum acquired in bbCID mode (C). The protonated
640 molecular ion was observed at *m/z* = 598.1630 (1), while the characteristic product ions were
641 observed at *m/z* = 556.1532 (2) and at *m/z* = 512.1271 (3). Proposed structures for the protonated
642 molecular ion and their product ions are depicted.

643 **Table 1.** Chlorophyll pigments identified by HPLC-PDA-ESI/APCI(+)-Q-TOF in this study.

	Pigment	t_R (min.)	UV-Vis maxima (nm)	$[M+H]^+$ (*m/z*) Calcd./Meas.	Elemental composition	Main MS^2 product ion	Reference
1	Chlorin p_6	4.8	399, 658	583.2551/583.2556	$C_{33}H_{34}N_4O_6$	-	-
2	Cu-13^2-hydroxy-pheophorbide *a*	6.9	400, 425,650	670.1847/670.1846	$C_{35}H_{34}CuN_4O_6$	520.0595 $[M+H-H_2O- C_2H_4O_2-C_5H_{12}]^+$	This work
3	Cu-pheophorbide *a*	7.8	400, 424,652	654.1897/654.1899	$C_{35}H_{34}CuN_4O_5$	594.1689 $[M+H-C_2H_4O_2]^+$	This work
4	Cu-pheophorbide *b*	8.8	440, 630	668.1690/668.1693	$C_{35}H_{32}CuN_4O_6$	608.1482 $[M+H-C_2H_4O_2]^+$	This work
5	Cu-pyropheophorbide *a*	9.9	404, 422,653	596.1842/596.1840	$C_{33}H_{32}CuN_4O_3$	568.1892 $[M+H-CO]^+$	This work
6	Lutein	10.8	424, 446, 474	569.4353/-[a]	$C_{40}H_{56}O_2$	551.4255 $[M+H-H_2O]^+$	Clarke et al. (1996)
7	15^1-hydroxy-lactone-pheophytin *a*	13.8	400, 668	903.5630/903.5633	$C_{55}H_{74}N_4O_7$	549.2507 $[M\text{-phytyl-CO-}CH_2O]^+$	Chen et al. (2015a)
8	ß-cryptoxanthin	14.2	431, 452, 479	553.4402/553.4404	$C_{40}H_{56}O$	535.4306 $[M+H-H_2O]$	De Rosso and Mercadante (2007)
9	13^2-hydroxy- pheophytin *b*	15.1	434, 653	901.5474/901.5470	$C_{55}H_{72}N_4O_7$	607.2541 $[M\text{+H-phytyl-}H_2O]^+$	Chen et al. (2015a)
10	Cu-pheophytin *b*	16.0	442, 632	946.4666/946.4667[b]	$C_{55}H_{70}CuN_4O_6$	662.2, 590.1 606.1323 $[M\text{+H-phytyl-}CO_2]^+$	Aparicio-Ruiz et al. (2011) This work
11	13^2-hydroxy-pheophytin *a*	17.0	410, 666	887.5682/887.5680	$C_{55}H_{74}N_4O_6$	593.2755 $[M\text{+H-phytyl-OH}]^+$	Chen et al. (2015a)
12	Cu-13^2-hydroxy-pheophytin *a*	21.8	400, 424, 655	948.4824/948.4825	$C_{55}H_{72}CuN_4O_6$	592.1528 $[M\text{+H-phytyl-CO}]^+$	This work
13	Cu-15^1-hydroxy-lactone-pheophytin *a*	23.2	412-644	964.4470/964.4468	$C_{55}H_{72}CuN_4O_7$	599.1474 $[M\text{+H-phytadiene-}C_3H_5NO_2]^+$	This work
14	pyropheophytin *b*	23.8	435, 655	827.5468/827.5471	$C_{53}H_{70}N_4O_4$	548 549.2489 $[M\text{+H-phytadiene}]^+$	Van Breemen et al. (1991) Chen et al. (2015a)

15	Cu-pheophytin *a*	24.3	400, 423, 652	932.4871/932.4874	$C_{55}H_{72}CuN_4O_5$	580.2 [M-CH_2CH_2COO-phytyl]	Aparicio-Ruiz et al. (2011)
						594.1688 [M+H-phytadiene-acetic acid]$^+$	This work
16	Cu-pyropheophytin *b*	24.6	444, 634	888.4607/888.4610	$C_{53}H_{68}CuN_4O_4$	608.1, 564.2, 536.1	Aparicio-Ruiz et al. (2011)
						582.1325 [M-phytadiene-C_2H_4] $^{\cdot+}$	This work
17	Cu-pheophytin *a´*	25.7	400, 423, 652	932.4871/932.4875	$C_{55}H_{72}CuN_4O_5$	580.2 [M-CH_2CH_2COO-phytyl]	Aparicio-Ruiz et al. (2011)
						594.1688 [M+H-phytadiene-acetic acid]$^+$	This work
18	Cu-pyropheophytin *a*	27.5	401, 424, 653	874.4818/874.4819	$C_{53}H_{70}CuN_4O_3$	596.1808 [M+H-phytadiene]$^+$	Pérez-Gálvez et al. (2015)
19	Phytyl-chlorin p_6	29.6	400, 660	861.5525/861.5528	$C_{53}H_{72}N_4O_6$	585.2710 [M+H-phytadiene]$^+$	This work

644 [a]The protonated molecular ion is barely observed due to in-source fragmentations of the main product ion. [b]Occasionally the adduct ion is observed as the main parent ion.

Table 2. Quantification of chlorophyll pigments (in percentage) in E141i-rich pellets and feces during the six weeks of experimentation. Identification is the same as detailed in Table 1.

Peak	Chlorophyll compound	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
		Pellet	Feces	Pellet	Feces	Pellet	Feces	Pellet	Feces	Pellet	Feces	Pellet	Feces
1	Chlorin p_6	0.83		0.85		0.87		0.81		0.80		0.86	
2	Cu-13^2-hydroxy-pheophorbide *a*	0.61		0.28		0.52		1.08		0.50		0.47	
3	Cu-pheophorbide *a*	1.17		0.40		0.74		0.63		1.50		0.94	
4	Cu-pheophorbide *b*	0.63		0.28		0.53		0.97		0.66		0.77	
5	Cu-pyropheoporbide *a*	4.58*	3.06	3.99	3.84	3.70	3.77	3.23	2.65	3.89	3.26	4.33*	3.62
9	13^2-hydroxy-pheophytin *b*	1.15	1.32	1.35	1.27	1.04	1.72	1.21	2.01*	1.08	1.68	1.62	1.13
10	Cu-pheophytin *b*	1.11	0.85	1.34	1.04	0.68	1.16	1.57	0.87	1.00	1.37	1.88	2.43*
12	Cu-13^2-hydroxy-pheophytin *a*	1.39	2.48*	1.15	1.45	1.08	2.08*	1.89	2.72	1.96	2.80	0.96	1.04
13	Cu-15^1-hydroxy-lactone-pheophytin *a*	5.92	5.75	5.01	5.10	6.37	7.24*	5.33	6.24*	5.33	5.68	5.85	5.13
14	pyropheophytin *b*	6.13	6.52	7.08	6.90	6.92	6.41	6.59	6.91	5.33	6.90*	6.58	7.02
15	Cu-pheophytin *a*	1.22	1.93	1.70	1.56	1.08	1.21	1.18	1.86*	1.79	1.49	1.21	1.33
16	Cu-pyropheophytin *b*	4.27	4.02	4.36	4.03	4.01	4.07	4.23	3.98	4.06	3.56	4.52	4.18
17	Cu-pheophytin *a´*	0.81	0.65	0.57	0.81	0.64	0.76	1.09	1.19	0.73*	1.01	0.68*	0.94
18	Cu-pyropheophytin *a*	69.31	72.84	70.78	73.04	70.88	71.11	69.34	70.68	70.47	71.35	68.44*	72.31
19	Phytyl-chlorin p_6	0.86	0.87	0.88	0.97	0.93	0.87	0.85	0.89	0.92	0.90	0.89	0.88

*Significant difference ($P < 0.05$) was observed with the corresponding paired value (pellet or feces), within each week.

Chlorophyll compound	Structure	R_1	R_2	R_3	R_4	R_5
Chlorin p_6	3	-	-	H	-	-
Cu-13^2-hydroxy-pheophorbide *a*	1	Cu^{+2}	CH_3	H	$COOCH_3$	OH
Cu-pheophorbide *a*	1	Cu^{+2}	CH_3	H	$COOCH_3$	H
Cu-pheophorbide *b*	1	Cu^{+2}	CHO	H	$COOCH_3$	H
Cu-pyropheoporbide *a*	1	Cu^{+2}	CH_3	H	H	H
15^1-hydroxy-lactone-pheophytin *a*	2	H	CH_3	Phytyl	$COOCH_3$	OH
13^2-hydroxy-pheophytin *b*	1	H	CHO	Phytyl	$COOCH_3$	OH
Cu-pheophytin *b*	1	Cu^{+2}	CHO	Phytyl	$COOCH_3$	H
13^2-hydroxy-pheophytin *a*	1	H	CH_3	Phytyl	$COOCH_3$	OH
Cu-13^2-hydroxy-pheophytin *a*	1	Cu^{+2}	CH_3	Phytyl	$COOCH_3$	OH
Cu-15^1-hydroxy-lactone-pheophytin *a*	2	Cu^{+2}	CH_3	Phytyl	$COOCH_3$	OH
pyropheophytin *b*	1	H	CHO	Phytyl	H	H
Cu-pheophytin *a*	1	Cu^{+2}	CH_3	Phytyl	$COOCH_3$	H
Cu-pyropheophytin *b*	1	Cu^{+2}	CHO	Phytyl	H	H
Cu-pyropheophytin *a*	1	Cu^{+2}	CH_3	Phytyl	H	H
Phytyl-chlorin p_6	3	-	-	Phytyl	-	-

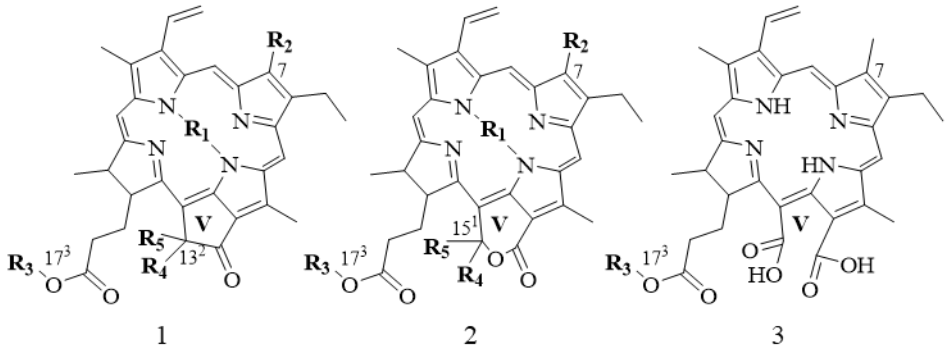
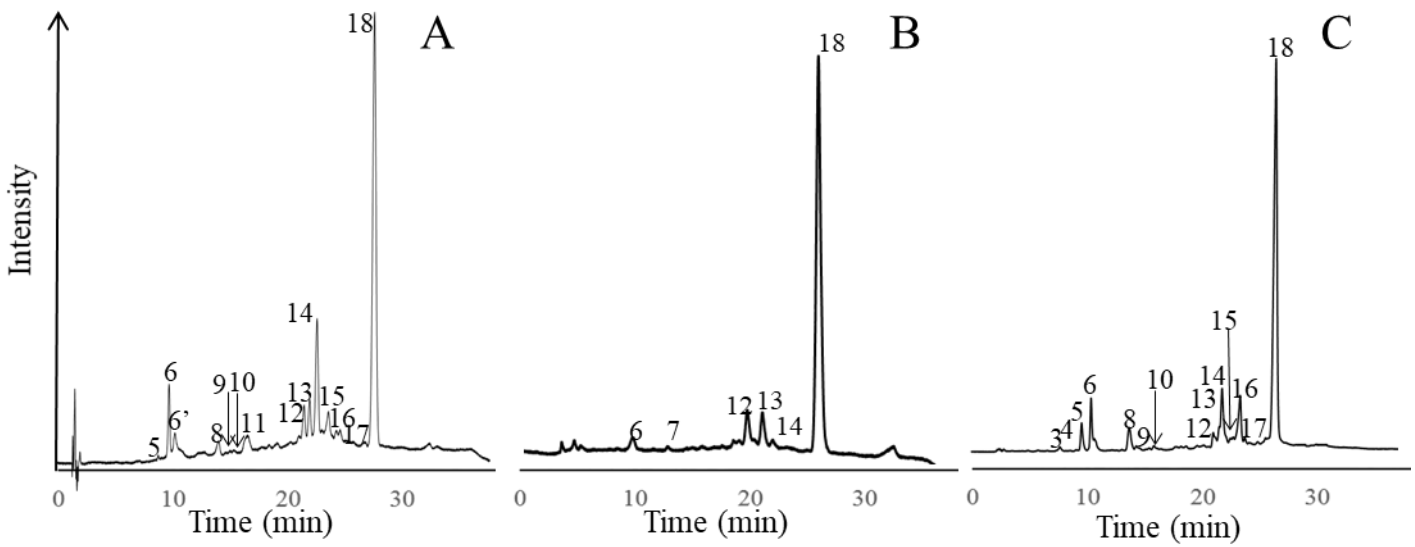


Figure 1-Pérez-Gálvez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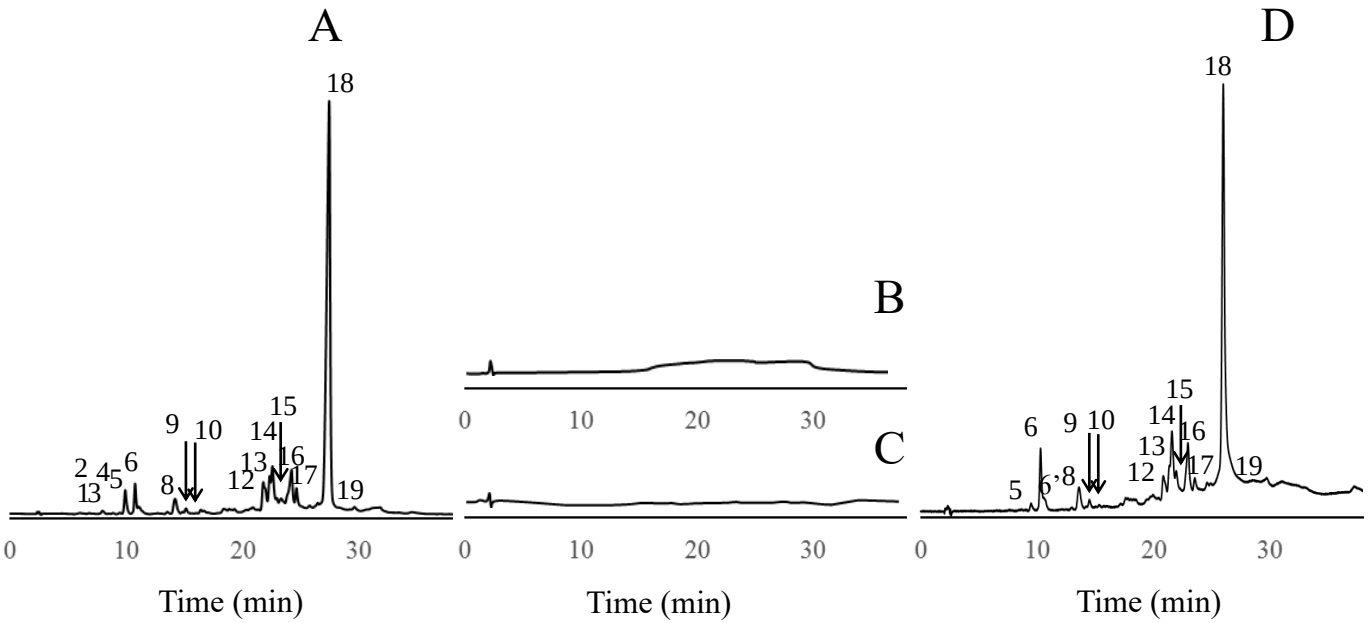
B
C
Intensity
Time (min)
Time (min)
Time (min)
0
10
20
30
5
6
6'
7
8
9
10
11
12
13
14
15
16
17
18

Figure 2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C
D
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15
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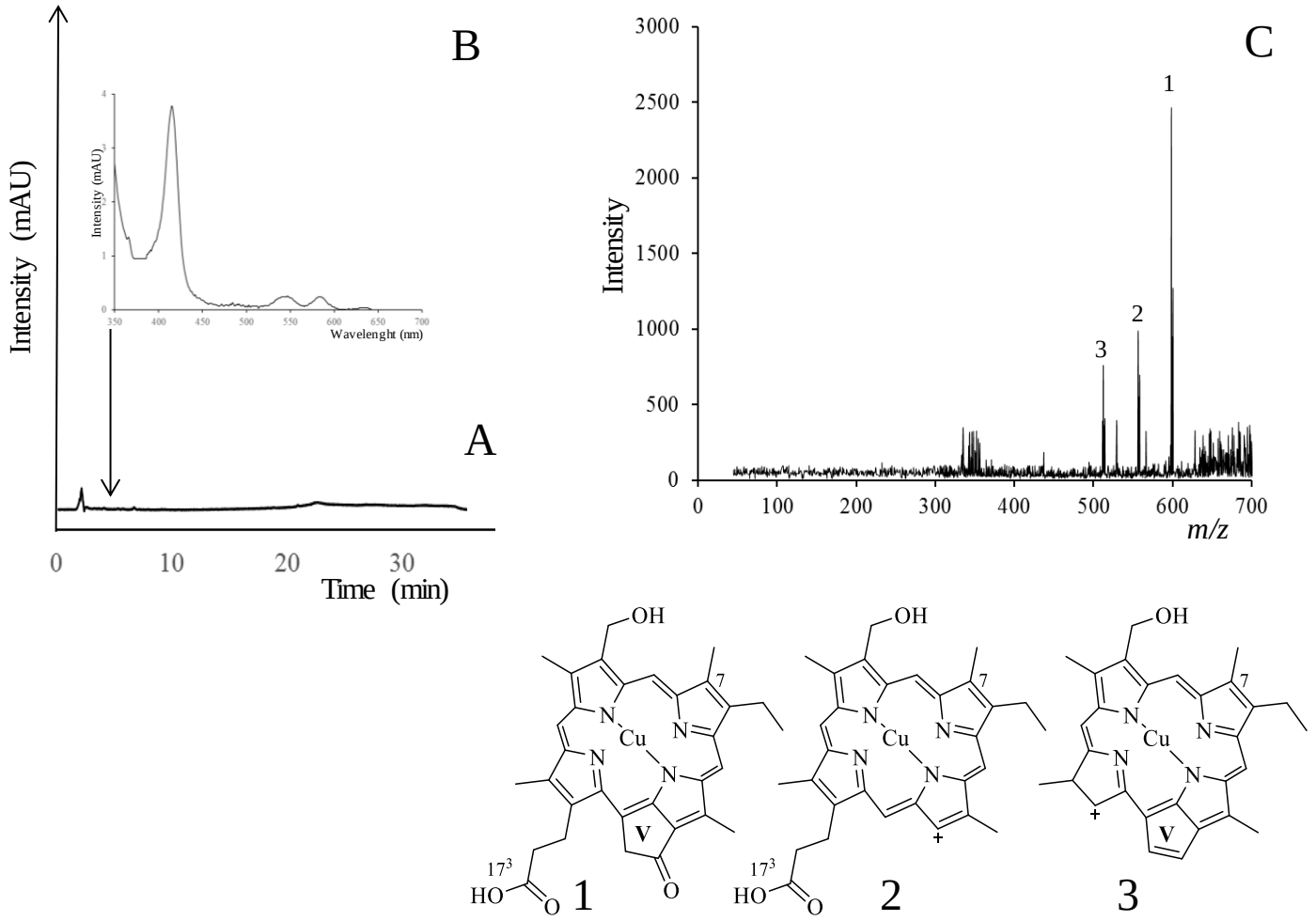
Time (min)
Time (min)
0
10
20
30


B
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Wavelenght (nm)
Intensity (mAU)
Time (min)
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20
30
C
Intensity
m/z
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2
3
OH
Cu
HO
O
1
2
3

Figure 4

678