**DEVELOPMENT OF AN ACCURATE AND HIGH-THROUGHPUT METHODOLOGY FOR
STRUCTURAL COMPREHENSION OF CHLOROPHYLLS DERIVATIVES. (II)
DEPHYTYLATED DERIVATIVES.**

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ABSTRACT

Dephytylated chlorophylls (chlorophyllides and pheophorbides) are the starting point of the chlorophyll catabolism in green tissues, components of the chlorophyll pattern in storage/processed food vegetables, as well as the favoured structural arrangement for chlorophyll absorption. In addition, dephytylated native chlorophylls are prone to several modifications of their structure yielding pyro-, 13²-hydroxy- and 15¹-hydroxy-lactone derivatives. Despite of these outstanding remarks only few of them have been analyzed by MSⁿ. Besides new protocols for obtaining standards, we have developed a new high throughput methodology able to determine the fragmentation pathway of sixteen dephytylated chlorophyll derivatives, elucidating the structures of the new product ions and new mechanisms of fragmentation. The new methodology combines, by first time, high resolution time-of-flight mass spectrometry and powerful post-processing software. Native chlorophyllides and pheophorbides mainly exhibit product ions that involve the fragmentation of D ring, as well as additional exclusive product ions. The introduction of an oxygenated function at E ring enhances the progress of fragmentation reactions through the β -keto ester group, developing also exclusive product ions for 13²-hydroxy derivatives and for 15¹-hydroxy-lactone ones. Consequently, while MS²-based reactions of phytylated chlorophyll derivatives point to fragmentations at the phytyl and propionic chains, dephytylated chlorophyll derivatives behave different as the absence of phytyl makes β -keto ester group and E ring more prone to fragmentation. Proposals of the key reaction mechanisms underlying the origin of new product ions have been made.

Keywords: API, chlorophyllide, fragmentation, HPLC, mass spectrometry, pheophorbide, standardized protocol.

1. Introduction

Chlorophylls are considered the most important pigments on Earth considering their essential role in photosynthesis during the light-harvesting process. The biosynthetic machinery of plant tissues is able to metabolise more than 10^9 tons of natural chlorophylls per year, a figure to note the wide distribution of this family of green pigments and the significance of the intermediates and metabolites derived from that process [1]. The first reaction steps of the chlorophyll breakdown pathway involve the enzymatic loss of the phytol chain [2] being chlorophyllides and pheophorbides key catabolites during the ripening/senescence of green tissues, or during storage/processing of foods, and the starting point of successive reactions implied in the catabolic pathway of chlorophyll. In addition, one of the few evidences to date related with the intestinal assimilation of chlorophylls in mammals shows that the dephytylation process is necessary to allow absorption of chlorophylls [3-4]. Consequently, it is essential to have a deep knowledge of the structural features of different dephytylated chlorophyll derivatives, to get accurate procedures to isolate the corresponding standards, as well as methodologies for unequivocal identification.

In this sense, natives chlorophyllides (dephytylated chlorophylls) and pheophorbides (Mg-free chlorophyllides) (Figure 1) are very sensitive to a diversity of oxidants, and consequently they form very easily two groups of oxidized compounds. One modification is the substitution of the hydrogen atom at C13² by a hydroxyl group, i.e. the 13²-hydroxy derivatives (Figure 1) and the second alteration implies the rearrangement of the isocyclic ring, setting up a lactone group, the 15¹-hydroxy-lactone derivatives (Figure 1). Finally, pyro-derivatives (pyro-chlorophyllides and pyro-pheophorbides) are formed due to the decarbomethoxylation at C13² position (Figure 1) in heated, canned or storage food materials. Chlorophyllide *a/b* and pheophorbide *a/b* are widely found in plant materials such as green vegetables or fruits [5-7], edible seaweeds [8], or natural herbs [9], the initial metabolites of chlorophyll degradation [2]. In heated, storage or canned food materials, pyro-pheophorbides are frequently present [9-10].

As their phytolated counterparts do, dephytylated chlorophylls show strong abilities of antioxidant activity towards free radicals, preventing lipid oxidation and oxidative damage [11-12] and potent anti-inflammatory activity *in vitro* [13]. Particularly, dephytylated chlorophylls (mainly pheophorbide *a/b* derivatives) are involved in the development of photodynamic therapy, where they behave as strong photosensitizers to catalyze the production of reactive oxygen species in cancer cells, when irradiated with a selected wavelength [14-16]. In addition to native pheophorbides, many *pheophorbide-like* compounds were identified in this research field, such as

pyro-pheophorbides and derivatives [17-18], as well as oxidized pheophorbides including 13²-hydroxy pheophorbide *a* and 15¹-hydroxy-lactone pheophorbide, arranged structurally as methyl ester [19].

Historically, the analysis of dephytylated chlorophylls has been limited to chlorophyllide, pheophorbide and pyro-pheophorbide. High performance liquid chromatography (HPLC) combined with MS have been applied to pigment separation and mass determination by means of different molecular ionization methods and *m/z* analyzers, including fast atom bombardment mass spectrometry (FAB-MS) [20-21], atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [9, 22-23] and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) [24]. However, data available of the product ions are limited to chlorophyllide and pheophorbide [21, 25].

The objective of this work is the development of a new high-throughput methodology able of determining the fragmentation pathways of sixteen dephytylated chlorophyll derivatives, and interpreting the possible structures of the new product ions and the novel mechanisms of fragmentation. To achieve this aim, it was necessary to develop new protocols to produce and isolate the standards of dephytylated chlorophyll derivatives, and furthermore to select more efficient ionization technique taking into account the chemical behaviour of the standards analyzed. The proposed methodology combines by first time, high resolution time-of-flight (hrTOF) mass spectrometry with a powerful post-processing software to the MSⁿ analysis of a complete set of dephytylated chlorophyll derivatives. The former measures the accurate mass of a selected molecular/product ion, while the latter allows prediction structural arrangement of new product ions not described so far. The blend of these useful tools helps to progress on the knowledge of the fragmentation pattern of dephytylated chlorophyll derivatives, compounds continuously present in our diet and, some of them intermediates of significant metabolic processes in plant tissues or arising from the absorption process in the gut of mammals.

2. Materials and methods

2.1 Reagents

Selenium dioxide (sublimed for synthesis, 98%), ammonium sulfate (99.5%), anhydrous sodium sulfate (99.0%) and silica gel 60 GF₂₅₄ for thin layer chromatography were supplied by Merck (Darmstadt, Germany). Potassium phosphate (98%), ammonium acetate (98%), Tris HCl, Triton X-100, sodium hydroxide, sodium chloride (99.5%), chlorophyll *a* and *b* were provided by

Sigma-Aldrich (St. Louis, MO, USA). Pheophorbide *a* was purchased from Wako Chemicals (Tokyo, Japan). Organic solvents and water (HPLC LC/MS grade) were supplied by Panreac (Barcelona, Spain). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). A tuning mix (ESI-L low concentration tuning mix, Agilent Technologies, Santa Clara, CA, USA) was used for MS calibration. Other reagents (chloroform, pyridine, diethyl ether, acetone, HCl, KCl, Na₂HPO₄, NaH₂PO₄, analysis grade) were supplied by Teknokroma (Barcelona, Spain).

2.2 Chlorophyll standards

2.2.1 Isolation of chlorophyll *a* and *b*

All the preparative work related to isolation of chlorophyll pigments was developed under green-light at 4 °C to avoid side enzymatic reactions. Chlorophyll *a* and *b* were isolated from spinach leaves (obtained in a local market) as follows. Fresh spinach leaves were cut and homogenized in acetone that was saturated with magnesium carbonate, then the green solution was collected by vacuum filtration and the vegetal residue was washed consecutively with acetone 2-3 times until it became yellowish. The acetone extracts were combined and mixed with a same volume of NaCl solution (10% w/v) in a separation funnel. Diethyl ether was added to transfer the pigments to the organic layer that once washed with Na₂SO₄ solution (2% w/v), was evaporated to dryness in a rotatory evaporator. Subsequently, pigments were recovered in acetone solution, filtered through nylon (0.45 µm) and subjected to thin layer chromatography (TLC) using silica gel (0.7 mm thickness layer). The developing solvent was light petroleum ether (65 °C-90 °C):acetone:diethylamine (10:4:1, v/v/v) [26]. Separated green bands corresponding to chlorophyll *a* and *b* were carefully scrapped from the TLC plate and solved in acetone. Immediately, chlorophyll *a* or *b* was collected and filtrated, and analyzed by HPLC-MS to obtain MS spectra that were compared with those obtained from commercial standards to ascertain their purity to carry out the following preparations for dephytylated chlorophyll compounds isolation.

2.2.2 Preparation of dephytylated chlorophyll pigments

Chlorophyllide *a* or *b* was obtained by enzymatic de-esterification of the corresponding parent chlorophyll using partially purified chlorophyllase from peels of mature *Citrus aurantium* (L.), fruits that show high activity of this enzyme [27]. Particularly, phosphate buffer (5 mM, pH

7.0) containing 50 mM KCl and 0.24% Triton X-100 [28] was used to extract chlorophyllase from homogenized peels squish with stirring for 1 h at 4 °C, and the crude enzymatic mixture was filtrated through three layers of gauze and subsequently subjected to centrifugation (5000 rpm, 10 min, 4°C). The supernatant was used to precipitate the enzyme protein. Four volumes of saturated (NH₄)₂SO₄ solution were added slowly into this enzyme solution with continuous stirring at 4 °C. Once the precipitation was achieved, the protein sediment was isolated by centrifugation (10000 rpm, 10 min, 4°C) and dissolved in phosphate buffer (100 mM, pH 7.0) to obtain a concentrated enzymatic solution for the following steps.

The enzymatic reaction mixture contains 100 mM Tris-HCl (pH 8.5), chlorophyll *a* or *b* dissolved in acetone and partially purified enzymatic extract in a 5:1:5 ratio [29] at 30 °C in shaking water-bath (85 cycles/min), during 45 min. The final volume was 26.4 mL for chlorophyllide *a* and 70.4 mL for chlorophyllide *b* to obtain ca. 3 mg or 1.5 mg, respectively. After this step, the reaction was finalized by adding four volumes of acetone. Chlorophyllide *a* or *b* was extracted with diethyl ether and evaporated to dryness in a rotatory evaporator and dissolved in acetone. The standards were kept at -20 °C until the MSⁿ analyses or for the production of other derivatives. Pheophorbide *a* and *b* were obtained by acidification of the corresponding chlorophyllides with 2-3 drops of 5 M HCl [30].

For preparation of 13²-hydroxy-, 15¹-hydroxy-lactone and pyro-dephytylated chlorophyll derivatives, method(s) and proper conditions of isolation were set up (section 3.1) [31]. 13²-Hydroxy- and 15¹-hydroxy-lactone- derivatives were obtained by selenium dioxide (7 mg/mL) oxidation of chlorophyllide *a/b* (or pheophorbide *a/b*) in heated pyridine solution under argon at 70 °C, during 3 h or 7 h, for 13²-hydroxy- and 15¹-hydroxy-lactone- derivatives, respectively. For the production of pyro-derivatives, parent compounds in pyridine solution blanketed with N₂ were heated at 80 °C for 4 h. The complete protocol of preparation and isolation procedures was performed in triplicate to obtain three batches of each standard that were subsequently analysed by HPLC-MSⁿ.

2.3 Liquid chromatography/Atmospheric-pressure ionization/Time-of-Flight Mass Spectrometry

The liquid chromatograph in the HPLC/APCI -(ionization source selected for the preliminary study)- or ESI-hrTOF-MS (ionization source selected for the definitive study) system was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed by injection of 30 µL of the standard isolation with the gradient described previously [32] but using 1 M ammonium acetate in water as the ion

reagent and 1.0 mL/min as flow rate. A stainless steel column (20 × 0.46 cm i.d.), packed with 3 µm C₁₈ Spherisorb ODS-2 (Teknokroma, Barcelona, Spain), was used. A split post-column of 0.25 mL/min was introduced directly on the mass spectrometer ESI or APCI source. Mass spectrometry was performed using a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with q-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an ESI or an APCI source. The scan range applied was m/z 50-1500 and mass resolving power was always over 18000 ($m/\Delta m$). Instrument was operated in positive ion mode. Mass spectra and data were acquired through broad-band Collision Induced Dissociation bbCID mode, providing MS and MS/MS spectra, simultaneously. Collision energy was estimated dynamically based on appropriate values for the mass and stepped across a +/- 10% magnitude range to ensure good quality fragmentation spectra. The instrument control was performed using Compass 1.3 for micrOTOF-Q II+Focus Option Version 3.0.

2.4 Data analysis

The *in-house* mass database created *ex professo* comprises monoisotopic masses, elemental composition and, optionally, retention time and characteristic fragment ions if known, for chlorophyllides, pheophorbides, and their derivatives compounds. Data evaluation was performed with Bruker Daltonics DataAnalysis 4.1, as described in our previous publications [31, 33]. The fragmentation schemes for a drawn molecular structure using fragmentation rules of mass spectrometry known in the literature, as well as the selected ionization mode and the number of fragmentation steps were obtained with the Mass Frontier™ 4.0 software.

3. Results and discussion

3.1 Preparation and isolation of dephytylated chlorophyll standards

Figure 1 shows the structures and numbering system of native chlorophyllides and pheophorbides *a* and *b*, and their oxidative derivatives identified in this work, present in vegetal tissues and food materials but with a less frequency than the parent chlorophylls. It is the first time that 10 standards of dephytylated chlorophyll derivatives have been produced and analyzed by MSⁿ. In addition, native compounds, chlorophyllide, pheophorbide, and pyropheophorbide *a/b* have been included for further investigation. Currently, commercial standards related to dephytylated chlorophyll pigments are limited to pheophorbide *a* and pyropheophorbide *a*,

consequently the initial substrates (chlorophyllide or pheophorbide *a/b*) in this work were derived from reactions except pheophorbide *a*, so that it was necessary to determine certain conditions for obtaining pure standards. The first one was to limit the content of the nonionic surfactant (Triton X-100), which is essential in the chlorophyllase reaction, but interferes in the MS analyses. Consequently, it was necessary to introduce in the protocol the precipitation with $(\text{NH}_4)_2\text{SO}_4$ (section 2.2.2) with the aim of removing the excess of Triton X-100. The second aspect was related to the purity of the enzymatic extract responsible for the chlorophyll dephytylation, as other active oxidative enzymes (peroxidases) during the dephytylation reaction that can react with chlorophylls forming unspecific oxidative derivatives. Table 1 shows the formation of 13^2 -hydroxy-chlorophyllides and 15^1 -hydroxy-lactone-chlorophyllide *a*. According to these results, the time of the chlorophyllase reaction has been reduced to 45 min, as chlorophyllase has higher affinity for chlorophyll than peroxidases [34].

As it is the first time to obtain oxidized dephytylated chlorophylls, the only references for the experimental conditions were those of the phytylated standards. Previous work have been made mainly with methanolic solvents, but then methoxy-derivatives are formed and not the intended hydroxy-derivatives. Consequently, for the 13^2 -hydroxy-derivatives the SeO_2 methodology was proved, as positive results have been obtained for phytylated chlorophylls [31, 35]. Several conditions (time and temperature) were checked to fit the best conditions, finally selecting 70 °C during 3 h. For the 15^1 -hydroxy-lactone production, another alternative protocol was used, that is, the oxidation with NaOH, but only presented positive results with chlorophyllide *a* (Table 1). After the study to obtain the best time and temperature conditions, 70 °C and 7 h were the selected ones for all the standards, according to final achieved concentration and the purity of the standard. Related to pyro-derivatives, literature refers also exclusively to phytylated derivatives, heating the solutions in pyridine at temperatures higher than 100 °C during unspecific time (from 1 to 24 h). As it is shown in Table 1, pyro-chlorophyllides are not produced at 100 °C, probably due to its higher labile character. Consequently, lower temperatures were preferred, selecting 80 °C during 4 h as the most appropriate conditions to obtain pyro-derivatives.

3.2 Type of ionization source applied for MS analysis of dephytylated chlorophyll derivatives

Up to now, the MS^2 analysis of chlorophyll derivatives has been a compendium of chlorophyll pigments, where most of analyzed derivatives are phytylated, and no specific assays have been developed for dephytylated compounds. Consequently, dephytylated chlorophyll standards have been always MS-analyzed by APCI [9, 20-24], except chlorophyllide *a* [25], that

is, with the same experimental MS configuration applied for the phytolated standards. One of our objectives was to decide whether the APCI ionization technique was appropriated or not for MS analysis of dephytylated chlorophylls derivatives, considering the higher polarity of this set of standards in comparison with the phytolated series, what may produce a different behaviour during the MS analyses. Figure 2 shows the MS² spectra of pheophorbide *a* obtained with different spray source techniques, APCI (Figure 2a) or ESI (Figure 2b). Although the molecular ion of pheophorbide *a* at m/z 593.2759 Da was clearly denoted in the APCI-MS² spectrum, we observed lower intensity values for the former as well as for the product ions that may come from it. On the other hand, the ESI-MS² spectrum is completely different, with high intensity values both for the molecular and for product ions. A similar behaviour was observed for the rest of pheophorbide *a/b* derivatives and for the chlorophyllides, and although we did not perform any modification in the acquisition parameters of the APCI source, to increase signal and quality of MSⁿ spectra. Based on these, the ESI ionization technique should be considered as the suitable one for MSⁿ analysis of dephytylated chlorophyll derivatives.

3.3 Analysis of dephytylated chlorophyll *a* and *b* derivatives by HPLC-ESI-hrTOF-MSⁿ

As the phytol chain is lacked in the dephytylated chlorophyll derivatives, the porphyrin ring is the shared core structure of the chlorophyllides and pheophorbides. Different oxygenated functions, located at the isocyclic ring (R₂ and R₃ in Figure 1), and/or the co-ordinated metal atom that may be present (chlorophyllide) or not (pheophorbide) (Figure 1), are combined to yield up to 16 dephytylated chlorophyll derivatives. Each isolated standard (8 chlorophyll derivatives from each family, *a* and *b*) was individually analysed by ESI high resolution-TOF-MS in positive mode and the results were studied with the assistance of the post-processing software, which provides a more accurate assignation of structural configuration of the different product ions. Table 2 shows the molecular weight and elemental composition for the dephytylated derivatives analyzed in this work, and the MS criteria obtained for compound identification, that is, accurate mass and error (ppm), and the mSigma value obtained with the SigmaFit™ algorithm. The consistency of the elemental composition and formulae of all product ions was possible performing the ESI-hrTOF-MS² analyses of chlorophyll derivatives in high resolution mode and with the assistance of the SmartFormula3D algorithm as described in Materials and Methods (section 2.4). Table 3 contains the relative abundance values (expressed in percentage term taking the intensity value of the most abundant product ion in each standard MS² analysis as 100) of the product ions produced from molecular ion. To smooth the process of identification of characteristic product

ions already described in literature [9, 20-25, 31, 36-37, 39], and the identification of specific ones that are singularly produced in a given compound, we used predictive software for *in silico* generation of product ions. This feature of the new proposed method allows identifying easily not only product ions in the MS² analyses, but also new ones not previously recognized.

Native chlorophyllides and pheophorbides, either from *a* and *b* family (Figure 3a) show the typical UV-visible spectrum for each compound. The maxima found were 432 nm and 664 nm for chlorophyllide *a*, 466 nm and 650 nm for chlorophyllide *b*, 410 nm and 666 nm for pheophorbide *a* and 436 nm and 654 nm for pheophorbide *b* [36-37]. Within all the native dephytylated chlorophyll compounds, only pheophorbide *a* has been extensively analysed by MS [20, 23-24], while some details of the MSⁿ behaviour of pheophorbide *b* [22], chlorophyllide *a* [9, 25] and chlorophyllide *b* [9] have been provided. The most complete study of the MS² characterization of native dephytylated chlorophyll compounds was made by van Breemen et al. (1991) by FAB-MS [21], finding similar product ions as those described for native phytylated chlorophylls. Our results for MS² analysis of native standards are presented in Table 3 (compounds I, V, IX and XIII), being the product ion derived from the fragmentation process at the C17propionic chain (product ion E, Table 3) the main one, with relative abundance values in the MS² spectra reaching 100% in all cases. This loss of 60.02 Da has been assigned previously to product ions B or E [21], but such fragmentation can be unambiguously assigned as product ion E with the use of the high resolution acquisition mode in combination with the assessment of predictive software. In this case, ionization takes place at the heteroatom of the carboxylic group with subsequent inductive cleavage involving oxygen functions and additional methylene groups. This product ion is also characteristic in the MS² spectra of phytylated chlorophyll derivatives. Its occurrence is very significant as it is the origin of new MS²-based reaction pathways for dephytylated chlorophyll derivatives, yielding new and consistent product ions noted as L, T, X and U in Table 3 and Figure 3b. The consistency of these product ions was checked with the SmartFormula3D algorithm, obtaining positive records in all cases. The *in silico* prediction points to this set of product ions that constitutes a reaction chain starting with ring D opening between C17 and C18 positions and following the reaction proposal depicted in Figure 4a. Product ions L arising from chlorophyllide and pheophorbide were first described by van Breemen et al. (1991) [21], although assigned to a different product ion (F-14 Da loss). The fragmentation process advances then to product ion T also present in all the native dephytylated chlorophylls. An additional fragmentation from E is product ion U, which means 102.06 Da loss for *a* series (C₇H₁₃O₄) or 114.03 Da loss for *b* series (C₇H₉O₅). The *in silico* prediction anticipates product ion U by means of fragmentation of the propionic chain, the β-keto ester group, as well as additional

losses at C8. Another common fragmentation process for all the native dephytylated chlorophyll derivatives is that one yielding product ion X, which means 148.07 Da loss ($C_6H_{11}O_4$) for *a* series or 162.05 Da loss ($C_6H_9O_5$) for *b* series. For product ion X several reaction processes are involved along the propionic chain, ring D opening, cleavage at the β -keto ester group and additional losses at C3 and C8. The product ions at m/z 467.1708 and 445.2023 were already described [21], although assigned to a different product ion (G-14 Da loss). Product ion noted as V in Table 3 is only present in pheophorbide *a* and *b* with a significant relative abundance value in both cases (22% and 78%, respectively). This product ion fits with a structural arrangement with opening of ring D, as in the product ion L, and loss of the β -keto ester function at C13². Dehydration is another exclusive fragmentation of Mg-free derivatives, as described before for pheophorbide *a* [20, 22]. The MS² spectra of native dephytylated chlorophyll derivatives did not show product ion G, without any other structural rearrangement in the molecule, as described for native phytylated chlorophyll derivatives [31]. In this case we have observed that the behaviour of the β -keto ester group at C13² is different. The progress of the MS²-based reactions after ionization at the heteroatom of the ester group develops, by inductive cleavage, an unstable carbocation, product ion M, (Table 3) with 32.02 Da loss from molecular ion that subsequently produces further product ions. In fact product ion S, present in all native dephytylated chlorophyll derivatives, could be considered as the final product ion of such reaction chain. Product ion S (with 106.02 Da loss) is compatible with a structure where the complete β -keto ester group is lost and the ring D is open in a similar fashion as happens in product ion L. As it was observed for the phytylated derivatives of the *b* series, the characteristic CO fragmentation from C13¹ (product ion noted as CO in Table 3) takes place in dephytylated *b* series. This characteristic fragmentation pattern encompasses the 13²-hydroxy- and pyro-derivatives of the *b* series, as it will be stated later, and the same fragmentation mechanism presented for the phytylated chlorophyll derivatives applies [31].

The 13²-hydroxy dephytylated derivatives (compounds II, VI, X and XIV in Table 3) show the same UV-Visible pattern as their native precursors, as it is supposed to be in parallel with the UV-Visible behaviour of the phytylated compounds, where the introduction of an hydroxyl function at C13² does not modify the features of the UV-visible spectrum. Figure 5a shows the MS² spectrum of 13²-hydroxy chlorophyllide *b*. The 13²-hydroxy dephytylated derivatives follow the same MS²-based reactions as their parent chlorophyllides and pheophorbides, without including the dissociation of the hydroxyl group, the main fragmentation processes described for the 13²-hydroxy phytylated derivatives [31]. Specifically, 13²-hydroxy dephytylated chlorophyll derivatives

present the product ions denoted in Table 3 retaining the hydroxyl function at C13², indicating that the loss involving that group is not thermodynamically favoured. Although product ion L is present in all the 13²-hydroxy dephytylated derivatives, it seems that the introduction of the hydroxyl group at C13² prevents further fragmentations from L (product ions T, U and X). The product ion E showed the highest relative abundance only in the case of 13²-hydroxy-chlorophyllide *a*, while the relative abundance values of that product ion was lower than 50% for the rest of hydroxyl derivatives, where product ions denoted as O or S showed the highest relative abundance values, so that the pathway to product ion S seems to be favored in comparison with the native forms. Product ions N and O are exclusively detected in 13²-hydroxy derivatives, where product ion N implies 34.04 Da loss while product ion O means 62.04 Da loss. These product ions are supposed to be intermediates between product ion M and product ion S, initial and end product ions of the reaction process depicted in Figure 4b. Since S and O are structures derived from M, there is the feasibility of this product ion to be figured out. Product ion M originates in the molecular ion after the loss of methanol that leaves the positive charge at C13³. The presence of a hydroxyl group at C13² increases the ability to form the carbocation M by means of the inductive effect produced by the oxygens of that hydroxyl group and the keto one located at C13³. In the case of native chlorophyllides and pheophorbides the inductive effect is lower as only the oxygen from keto group may produce this effect, which has been reproduced in Figure 6. The feasibility of producing product ion M in hydroxyl derivatives has a further consequence. Product ion V that may derive from M one was observed even for 13²-hydroxy-chlorophyllides and at higher intensities for pheophorbides, but chlorophyllides did not show this product ion. For *b* series of 13²-hydroxy-derivatives it was detected the characteristic product ion where CO from isocyclic ring has been lost, with relative abundance values below 5%, as stated for the phytolated chlorophyll derivatives [31]. As observed for native parent compounds, dehydration is exclusive of 13²-hydroxy- pheophorbides.

The introduction of a lactone group at C15¹ (Figure 1) in phytolated chlorophylls implies a bathochromic shift of 10 nm of the Soret band [38], so that the same effect can be applied to 15¹-hydroxy-lactone dephytylated chlorophylls. In fact for 15¹-hydroxy-lactone chlorophyllide *a* the UV-visible maxima are 420 nm and 656 nm, for 15¹-hydroxy-lactone chlorophyllide *b* are 454 nm and 654 nm, for 15¹-hydroxy-lactone pheophorbide *a* are 400 nm and 666 nm, and for 15¹-hydroxy-lactone pheophorbide *b* are 420 nm and 654 nm. The ESI-MS² spectra of 15¹-hydroxy-lactone dephytylated derivatives (compounds III, VII, XI and XV in Table 3) show product ions, D and E, in agreement with native and 13²-hydroxy-dephytylated chlorophylls as well as phytolated chlorophylls [31]. In addition, new, consistent, and exclusive product ions are detected in this set

of chlorophyll standards, denoted as P, Q and R in Table 3. Figure 5b shows the MS² spectrum of 15¹-hydroxy-lactone chlorophyllide *b*. Although the relative abundance of the common product ions generated by fragmentation of propionic chain (D, E, L) was not remarkable, except for product ions E and L in 15¹-hydroxy-lactone chlorophyllide *b*, the new set of product ions previously mentioned reached relative abundance values from 16% to 100%. The latter relative abundance value was reached in the case of product ion R and for all 15¹-hydroxy-lactone derivatives. R fragmentation implies 87.01 Da loss and this product ion resembles the structural configuration of product ions described for phytylated chlorophyll derivatives [39] and that were noted as K/K', an exclusive fragmentation for 15¹-hydroxy-lactone phytylated chlorophylls [31]. This product ion comes from the loss of C₃H₃O₃, fragmentation that takes place at the β-keto ester group including the C15¹ carbon atom. The fragmentation scheme for generation of R consists on ionization of the molecular ion at the double bond between C14 and C15 positions, then inductive cleavage of the C15-C15¹ bond and charge migration to the lactone group, and subsequent fragmentation of the hydroxyl and β-keto-ester functions. Consequently it implies the opening of the isocyclic ring. MS²-based reactions progress in product ion R and originate two additional product ions. Product ion P is also exclusively detected in this set of dephytylated chlorophyll standards and implies the 72.02 Da loss, and it is produced by means of the breakdown of isocyclic ring in a similar fashion as described above but including the lactone function in the fragmentation profile. Product ion Q, 90.03 Da loss, derives from P by means of an additional dehydration from the propionic chain. The consistency of the product ions has been checked, and the exact mass and elemental composition matched with our requirements (section 2.4). As well as stated before for the phytylated lactone chlorophyll derivatives, direct fragmentation of the CO group at the C ring in *b* series is not observed for 15¹-hydroxy-lactone dephytylated chlorophylls [31]. This finding supports our hypothesis about the origin of the CO group breakdown in native and hydroxyl *b* series derivatives, arising from the fragmentation of the keto group at C13¹ and not from the aldehyde group at C7, following our mechanism proposal [31]. The presence of a 15¹-hydroxy-lactone group hinders the fragmentations yielding product ions T and U, as in the 13²-hydroxy derivatives, but does not block the progress to product ion X, as this is always present in the MS² analyses of 15¹-hydroxy-lactone dephytylated chlorophylls. In addition, and in consonance with results mentioned above, dehydration is only present in 15¹-hydroxy-lactone pheophorbides. It is important to highlight that there are no referenced data about the chemical behaviour of 13²-hydroxy- and 15¹-hydroxy-lactone dephytylated derivatives during MS² analysis, so that the agreements and consistency among these results and the one observed for the native series support our observations.

References about MSⁿ analyses of pyro-derivatives are reduced to pyro-pheophorbides. Thus, the work of Gauthier-Jaques (2001) [22] makes some indications about the MS behaviour of pyro-pheophorbide *a* and *b* and even includes some MS² product ions although any hypothesis about their elemental composition and structural rearrangement is made. The UV-visible spectrum of the pyro-dephytylated chlorophylls shows the typical maxima: 432 nm and 664 for pyro-chlorophyllide *a*, 460 nm and 654 nm for pyro-chlorophyllide *b*, 410 nm and 666 nm for pyro-pheophorbide *a*, and 438 nm and 654 for pyro-pheophorbide *b* [32, 37]. The simple structural arrangement of pyro-derivatives (Figure 1) in comparison with the rest of dephytylated chlorophyll derivatives makes the MS² spectra of these derivatives content fewer product ions than their native and oxygenated counterparts. Thus, the main product ions (IV, VIII, XII and XVI compounds in table 3) either for *a* and *b* series are D for Mg-pyro derivatives, reaching 100% of relative abundance values, and L for Mg-free-pyro derivatives. Interesting is the fact that the characteristic product ion E was not observed in these derivatives, a remarkable similarity with the phytylated derivatives [31]. Product ion noted as CO in Table 3 was exclusively observed in *b* series and at higher relative intensity values than those observed for the rest of dephytylated derivatives of the *b* series (with the exception of 15¹-hydroxy-lactone derivatives that do not follow this fragmentation process). Lastly, and in accordance with the rest of dephytylated chlorophyll compounds analyzed, only Mg-free derivatives showed the dehydration process.

4. Conclusions

The application of an effective methodology, hyphenation of high resolution MS technique with the assessment of predictive software, has allowed the appropriate identification of several product ions and the establishment of new fragmentation pathways, which are characteristic of specific oxidations/modifications of dephytylated chlorophyll derivatives. Native chlorophyllides and pheophorbides mainly exhibit product ions that involve the fragmentation of D ring, as well as additional new and exclusive product ions (T and U). The introduction of an oxygenated function at E ring enhances the progress of fragmentation reactions through the β -keto ester group, developing also new and exclusive product ions for 13²-hydroxy derivatives (N and O) and for 15¹-hydroxy-lactone derivatives (P, Q and R). Only Mg-free derivatives undergo dehydration process, while *b* series derivatives exhibit the CO fragmentation, excluding lactone derivatives, as observed for the phytylated *b* series derivatives and supporting our hypothesis of CO fragmentation from C13¹. While phytylated chlorophyll derivatives yield product ions from phytyl and propionic chains fragmentation, dephytylated chlorophyll derivatives show different reaction

sites for MS²-based reactions, as the absence of phytol makes β -keto ester group and E ring more prone to fragmentation. In fact, we have checked the absence of such fragmentations in phytylated chlorophylls, showing that presence of phytol avoids opening of E ring. Taking into account the chlorophyll degradation pathways in green tissues and the most likely route of chlorophyll assimilation, it is essential to have a precise characterization of all possible dephytylated chlorophyll derivatives, as well as a thoughtful analysis of the progress of the MS²-based reactions for all of them.

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Figure legends

Figure 1.- Structural configuration, names and numbering system for parent chlorophyllide *a* and *b*, and their Mg-free derivatives originated by different protocols and analyzed by HPLC-ESI-hrTOF-MSⁿ.

Figure 2.- MS² spectrum of pheophorbide *a* obtained by atmospheric pressure chemical ionization (a) or electrospray ionization (b). Identification of MS² product ions is as follows: Figure 2b, 1: 575.2664; 2: 561.2466; 3: 547.2603; 4: 533.2504; 5: 519.2362; 6: 505.2213; 7: 487.2432; 8: 477.2329; 9: 447.2153; 10: 445.2023; 11: 431.1858.

Figure 3.- Sites of fragmentation occurring during HPLC-ESI-hrTOF-MS² analysis of dephytylated 13²-hydroxy, 15¹-hydroxy-lactone, and pyro- derivatives (b, c, d, respectively) and their parent chlorophyllides/pheophorbides (a) following the labelling system discussed by Van Breemen et al., (1991). For structural identification of radicals R1 to R4 and numbering system see Figure 1.

Figure 4.- Proposed fragmentation mechanisms: (a) Fragmentation pathway of the product ion E derived from pheophorbide *a*, to yield product ion L that involves ring opening by inductive cleavage between C17 and C18 positions, subsequent hydrogen rearrangement, and inductive cleavage. (b) Fragmentation pathway of the product ion M derived from 13²-hydroxy-pheophorbide *a*, to yield product ion S that involves isocyclic ring opening by inductive cleavage subsequent hydrogen rearrangement (product ion N), CO loss and hydrogen rearrangement (product ion O) and final CO loss (product ion S).

Figure 5.- MS² spectrum of 13²-hydroxy chlorophyllide *b* (Figure 5a) and 15¹-hydroxy-lactone chlorophyllide *b* (Figure 5b) obtained by electrospray ionization high resolution time-of-flight-MS². Identification of MS² product ions is as follows: Figure 5a, 1: 617.2181; 2: 613.1965; 3: 599.2155; 4: 595.1830; 5: 585.1975; 6: 567.1852; 7: 557.2029; 8: 539.1925; 9: 485.1833. Figure 5b, 1: 615.2065; 2: 601.1863; 3: 574.2065; 4: 573.1964; 5: 557.1992; 6: 539.1895; 7: 501.1774. A black diamond marks the molecular ion at *m/z* 645.2186 and 661.2117 for 13²-hydroxy chlorophyllide *b* and 15¹-hydroxy-lactone chlorophyllide *b*, respectively.

604 **Figure 6.-** Proposal of the inductive effect accounted for native and hydroxyl dephytylated
605 chlorophyll derivatives once the carbocation M has been released from molecular ion.

Figure 1

FIGURE 1

Compound	Structure	Mg ^a	R ₁	R ₂	R ₃	R ₄
Chlorophyllide <i>a</i>	1	+	CH ₃	H	COOCH ₃	hydrogen
13 ² -Hydroxy-chlorophyllide <i>a</i>	1	+	CH ₃	OH	COOCH ₃	hydrogen
15 ¹ -Hydroxy-lactone chlorophyllide <i>a</i>	2	+	CH ₃	OH	(15 ²)COOCH ₃	hydrogen
Pyro-chlorophyllide <i>a</i>	1	+	CH ₃	H	H	hydrogen
Chlorophyllide <i>b</i>	1	+	CHO	H	COOCH ₃	hydrogen
13 ² -Hydroxy chlorophyllide <i>b</i>	1	+	CHO	OH	COOCH ₃	hydrogen
15 ¹ -Hydroxy-lactone chlorophyllide <i>b</i>	2	+	CHO	OH	(15 ²)COOCH ₃	hydrogen
Pyro-chlorophyllide <i>b</i>	1	+	CHO	H	H	hydrogen
Pheophorbide <i>a</i>	1	-	CH ₃	H	COOCH ₃	hydrogen
13 ² -Hydroxy pheophorbide <i>a</i>	1	-	CH ₃	OH	COOCH ₃	hydrogen
15 ¹ -Hydroxy-lactone pheophorbide <i>a</i>	2	-	CH ₃	OH	(15 ²)COOCH ₃	hydrogen
Pyro-pheophorbide <i>a</i>	1	-	CH ₃	H	H	hydrogen
Pheophorbide <i>b</i>	1	-	CHO	H	COOCH ₃	hydrogen
13 ² -Hydroxy pheophorbide <i>b</i>	1	-	CHO	OH	COOCH ₃	hydrogen
15 ¹ -Hydroxy-lactone pheophorbide <i>b</i>	2	-	CHO	OH	(15 ²)COOCH ₃	hydrogen
Pyro-pheophorbide <i>b</i>	1	-	CHO	H	H	hydrogen

^afor pheophorbide derivatives Mg atom is substituted by two hydrogen atoms

Figure 2

FIGURE 2

Figure 3

FIGURE 3

Figure 4

FIGURE 4

Figure 5

FIGURE 5

Figure 6

FIGURE 6

Table 1.- Conditions applied for the preparation of dephytylated chlorophyll derivatives standards and subsequent analysis by HPLC-ESI-hrTOF-MSⁿ.

	13 ² -Hydroxy-derivatives			15 ¹ -Hydroxy-lactone-derivatives			Pyro-derivatives	
	Enzyme extract	70°C 3 h	100°C 1 h	0.5% NaOH	SeO ₂ oxidation	Enzyme extract	80°C 4h	100°C 4h
Chlorophyllide <i>a</i>	+	+	-	+	+	+	+	-
Chlorophyllide <i>b</i>	+	+	-	-	+	n.d.	+	-
Pheophorbide <i>a</i>	n.d.	+	-	-	+	n.d.	+	+
Pheophorbide <i>b</i>	n.d.	+	+	-	+	n.d.	+	+

Enzyme extract means chlorophyllase incubation.
Pyro-derivatives were isolated from a heated chlorophyllide/pheophorbide solution in pyridine.
13²-hydroxy-derivatives were isolated from a heated chlorophyllide/pheophorbide solution containing 7 mg/mL SeO₂.
15²-hydroxy-lactone-derivatives were isolated from a chlorophyllide/pheophorbide solution oxidized with 0.5% NaOH in an open vessel for 5-10 min or heating at 70 °C for 7 h with SeO₂.
ⁿ *Stands for negative or positive results, respectively in the MS analysis of the isolated intended standard applying the criteria for mass accuracy and SigmaFit values (mass error< 5 ppm, SigmaFit< 50).
n.d. not determined.

Table 2.- Retention time and high accuracy measurements of dephytylated chlorophyll standards derivatives analyzed by ESI-hrTOF-MSⁿ.

Compound	<i>t_R</i> (min)	Error (ppm)	mSigma	Molecular formula	[M+H] ⁺ (<i>m/z</i>)	
					MW. calc.	MW. meas.
Chlorophyllide <i>a</i>	7.2	4.9	28.5	C ₃₅ H ₃₄ MgN ₄ O ₅	615.2452	615.2422
13 ² -Hydroxy-chlorophyllide <i>a</i>	5.0	4.5	19.9	C ₃₅ H ₃₄ MgN ₄ O ₆	631.2402	631.2367
15 ¹ -Hydroxy-lactone-chlorophyllide <i>a</i>	4.0	3.6	26.2	C ₃₅ H ₃₄ MgN ₄ O ₇	647.2351	647.2327
Pyro-chlorophyllide <i>a</i>	6.8	2.1	35.6	C ₃₃ H ₃₂ MgN ₄ O ₃	557.2398	557.2395
Chlorophyllide <i>b</i>	5.3	2.7	18.5	C ₃₅ H ₃₂ MgN ₄ O ₆	629.2245	629.2241
13 ² -Hydroxy-chlorophyllide <i>b</i>	3.1	1.2	14.6	C ₃₅ H ₃₂ MgN ₄ O ₇	645.2194	645.2186
15 ¹ -Hydroxy-lactone chlorophyllide <i>b</i>	2.4	4.0	31.9	C ₃₅ H ₃₂ MgN ₄ O ₈	661.2143	661.2117
Pyro-chlorophyllide <i>b</i>	4.1	0.1	38.2	C ₃₃ H ₃₀ MgN ₄ O ₄	571.2190	571.2191
Pheophorbide <i>a</i>	7.9	4.8	11.8	C ₃₅ H ₃₆ N ₄ O ₅	593.2759	593.2711
13 ² -Hydroxy-pheophorbide <i>a</i>	6.8	0.3	12.1	C ₃₅ H ₃₆ N ₄ O ₆	609.2708	609.2705
15 ¹ -Hydroxy-lactone pheophorbide <i>a</i>	8.1	1.8	23.5	C ₃₅ H ₃₆ N ₄ O ₇	625.2657	625.2645
Pyro-pheophorbide <i>a</i>	9.6	4.9	22.4	C ₃₃ H ₃₄ N ₄ O ₃	535.2704	535.2678
Pheophorbide <i>b</i>	6.5	0.3	9.2	C ₃₅ H ₃₄ N ₄ O ₆	607.2557	607.2549
13 ² -Hydroxy- pheophorbide <i>b</i>	5.2	3.5	47.3	C ₃₅ H ₃₄ N ₄ O ₇	623.2500	623.2522
15 ¹ -Hydroxy-lactone pheophorbide <i>b</i>	4.3	2.1	32.6	C ₃₅ H ₃₄ N ₄ O ₈	639.2449	639.2439
Pyro- pheophorbide <i>b</i>	7.7	3.6	38.9	C ₃₃ H ₃₂ N ₄ O ₄	549.2496	549.2476

MW. Cal. means molecular weight calculated for [M+H]⁺. MW meas. means molecular weight measured for [M+H]⁺. Error and mSigma correspond to high accuracy measurements of each chlorophyll standard derivative that should be within the tolerance limits (<±5 ppm and <50, respectively), performed by DataAnalysis 4.1 [40].

Table 3.- Product ions (m/z) in the ESI-hrTOF-MS² spectra of dephytylated chlorophyll derivative standards.

Compound ^a	CO	D	E	F	L	T	U	X	M	N	O	S	V	P	Q	R	H ₂ O
I		569.2466 (1) ^b	555.2215 (100)		527.2245 (7)	500.2166 (3)	453.1554 (26)	467.1708 (38)				509.2186 (3)					
V	601.2302 (2)	583.221 (1)	569.2018 (100)		541.2068 (29)	513.1811 (6)	455.1678 (1)	467.1698 (31)	597.1948 (1)			523.1963 (4)					
IX		547.2603 (1)	533.2504 (100)	519.2362 (7)	505.2213 (41)	477.2329 (4)	431.1858 (12)	445.2023 (14)	561.2466 (2)			487.2432 (4)	447.2153 (22)				575.2664 (1)
XIII	579.2597 (54)	561.2436 (8)	547.2331 (100)		519.2353 ^c (80)	491.2108 (60)	433.2020 (33)	445.2048 (29)	575.2295 (4)			501.2275 (21)	447.2172 (78)				589.2586 (2)
II		585.2297 (53)	571.2158 (100)		543.2119 (57)				599.2165 (8)	581.2028 (27)	553.2024 (78)	525.2092 (32)	485.1766 (5)				
VI	617.2181 (1)	599.2155 (6)	585.1975 (25)		557.2029 (15)				613.1965 (1)	595.1830 (5)	567.1852 (100)	539.1925 (67)	485.1833 (9)				
X		563.2585 (10)	549.2496 (44)	535.2339 (39)	521.2422 (59)				577.2461 (14)	559.2339 (70)	531.2381 (69)	503.2420 (100)	463.2138 (41)				591.2604 (31)
XIV	595.2534 (5)	577.2453 (11)	563.2390 (11)	549.2187 (9)	535.2314 ^c (38)				591.2246 (6)	573.2128 (41)	545.2108 (64)	517.2221 (100)	463.2134 (38)				605.2382 (11)
III		601.2363 (17)	587.2101 (6)					499.1971 (11)						543.2163 (10)	525.2077 (30)	560.2301 (100)	
VII		615.2066 (5)	601.1863 (2)		573.1964 (5)			501.1774 (45)						557.1992 (16)	539.1895 (21)	574.2065 (100)	
XI		579.2537 (3)	565.2430 (2)					477.2268 (7)						521.2401 (5)	503.2331 (4)	537.2451 (100)	607.2492 (5)
XV		593.2306 (14)	579.2359 (37)		551.2256 ^c (26)			479.2061 (48)						535.2362 (50)	517.2250 (53)	552.2359 (100)	621.2412 (14)
IV		511.2310 (100)			469.1889 ^d (65)												
VIII	543.2246 (65)	525.2103 (100)			483.1696 ^d (39)												
XII		489.2549 (3)		461.2299 (15)	447.2159 ^d (100)												517.2641 (1)
XVI	521.2543 (86)	503.2371 (8)		475.2099 (26)	461.1999 ^d (100)												531.2314 (4)

^a, I: chlorophyllide *a*; II: 13²-hydroxy-chlorophyllide *a*; III: 15¹-hydroxy-lactone- chlorophyllide *a*; IV: pyro-chlorophyllide *a*; V: chlorophyllide *b*; VI: 13²-hydroxy- chlorophyllide *b*; VII: 15¹-hydroxy-lactone-chlorophyllide *b*; VIII: pyro-chlorophyllide *b*; IX: pheophorbide *a*; X: 13²-hydroxy- pheophorbide *a*; XI: 15¹-hydroxy-lactone- pheophorbide *a*; XII: pyro- pheophorbide *a*; XIII: pheophorbide *b*; XIV: 13²-hydroxy- pheophorbide *b*; XV: 15¹-hydroxy-lactone-pheophorbide *b*; XVI: pyro-pheophorbide *b*.

^b, Relative intensities for each m/z value appear in parentheses and are expressed as a percentage of the most abundant fragment ion in each standard.

^c, For these product ions the error is between 5 and 7 ppm.

^d, Product ion L (depicted in Figure 4a) does not include the carbomethoxy group in this case.