DEVELOPMENT OF AN ACCURATE AND HIGH-THROUGHPUT METHODOLOGY FOR STRUCTURAL COMPREHENSION OF CHLOROPHYLLS DERIVATIVES. (I) PHYTYLATED DERIVATIVES.

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ABSTRACT

Phytylated chlorophyll derivatives undergo specific oxidative reactions through the natural metabolism or during food processing or storage, and consequently pyro-, 13^2-hydroxy-, 15^1-hydroxy-lactone chlorophylls and pheophytins (*a* and *b*) are originated. New analytical procedures have been developed here to reproduce controlled oxidation reactions that specifically, and in reasonable amounts, produce those natural target standards. At the same time and under the same conditions, sixteen natural chlorophyll derivatives have been analyzed by APCI-HPLC-hrMS^2 and most of them by the first time. The combination of the high resolution MS mode with powerful post-processing software has allowed the identification of new fragmentation patterns, characterizing specific product ions for some particular standards. In addition, new hypotheses and reaction mechanisms for the established MS^2-based reactions have been proposed. As a general rule, the main product ions involve the phytyl and the propionic chains but the introduction of oxygenated functional groups at the isocyclic ring produces new and specific product ions and at the same time inhibits some particular fragmentations. It is noteworthy that all *b* derivatives, except 15^1-hydroxy-lactone compounds, undergo specific CO losses. We propose a new reaction mechanism based in the structural configuration of *a* and *b* chlorophyll derivatives that explain the exclusive CO fragmentation in all *b* series except for 15^1-hydroxy-lactone *b* and all *a* series compounds.

Keywords: chlorophylls, fragmentation, HPLC, mass spectrometry, pheophytins, standarised protocol.

1. INTRODUCTION

Due to their essential roles in photosynthesis, chlorophylls are omnipresent from algae to higher plants. Such vital function derives from its structure, chlorophylls can absorb light quanta effectively but also they can release and take up electrons reversibly by means of their aromatic 18 π–electron (18)-diazaannulene macrocycle. Additionally, these photochemical compounds have been also proved to possess prominent benefits to human health when consumed along with vegetable products or edible seaweeds in our daily diet, such as the antimutagenic effect [1-2], antigenotoxic properties [3], and potent antioxidant capacity to scavenge free radicals and to prevent lipid oxidation [4]. However, many further studies were hampered by a lack of an overall structural analysis of all the chlorophyll derivatives that emerge in biological metabolism, tissue senescence or during food processing.

Besides the components of the photosynthetic chain (chlorophyll *a* and *b*, and pheophytin *a*), a number of complex chlorophyll derivatives are formed in green tissues as a consequence of the natural metabolism or during the food storage/processing. Among the chlorophyll derivatives that keep the phytyl chain, there are four main groups of chlorophyll derivatives. Pheophytins (Figure 1) are formed when the central magnesium atom of the tetrapyrrol ring is easily replaced by two hydrogen atoms, as a consequence of metal chelating substance reaction during the chlorophyll catabolism in the leaf senescence or fruit ripening [5], or during cooking [6] or food processing [7-9]. Secondly, pyro-derivatives (pyro-chlorophylls and pyro-pheophytins) are formed due to the decarbomethoxylation at C-13^2 position (Figure 1) in heated, canned or storage food materials, being these compounds frequently found in canned and boiled peas [10], in chinese herbs [11-12], or in olive oils [13]. Finally, native chlorophyll and pheophytins are very sensitive to a variety of oxidants and consequently they form very easily two groups of oxidized compounds. One modification is the substitution of the H atom at C13^2 by an hydroxyl group, the so-called 13^2-hydroxy derivatives (Figure 1) that have been identified in senescent plant tissues or in dried herb products [11-12, 14-16]. The second alteration implies the rearrangement of the isocyclic ring through the formation of a lactone group, the 15^1-hydroxy-lactone derivatives (Figure 1), found in different tissues [16-18]. Nevertheless, since 1913 the term "allomerized" refers to the oxidation of chlorophyll by triplet oxygen in alcohol solution [19]. Among the allomerization products identified and studied in depth are 15^1-methoxy-lactone-, 13^2-methoxy-, and 13^2-hydroxy-chlorophyll. Although outstanding contributions have dealt with the MS/MS characterization of allomerized chlorophylls [20-22], it is important to highlight that 15^1-methoxy-lactone- and 13^2-methoxy-chlorophyll derivatives are not produced *in vivo*, as naturally

chlorophyll compounds are not in contact with alcoholic solutions, so the natural substituent in chlorophyll derivatives is the hydroxyl group, forming 13^2-hydroxy- or 15^1-hydroxy-lactone chlorophyll derivatives. In fact, the common technique to produce the 13^2-hydroxy chlorophyll *a* and *b* used for MS analyses is the methanolic allomerization [21-24] which leads to non-specific oxidation and calls for large volumes of initial substrates, because the hydroxyl derivatives are produced in minor amounts. Coming to the oxidation to lactone group, traditional methods including exposing chlorophyll solutions in methanol to air [22-26] or exceptionally alkaline treatment [27] can only guarantee the yield of 15^1-methoxy-lactone chlorophyll but not 15^1-hydroxy-lactone counterpart, which is the particular compound present in natural food resources or physiologic tissues. Considering the lack of protocols to produce conveniently the natural chlorophyll derivatives with hydroxyl function at $C13^2$ or at $C15^1$ (lactone chlorophyll derivatives), it is of great importance to establish specific reactions pathways to produce a concrete oxidation and to develop new methods to obtain specific chlorophyll derivative standards.

High performance liquid chromatography (HPLC) coupled to UV-vis detection is the most frequent method to effectively separate and analyse chlorophyll derivatives. However, taking into account the abundance of such compounds, some neighbouring peaks hold indistinguishable UV spectrum, which makes the identification more complicated especially when the native chlorophyll mixture contains epimers, allomerized or de-carbomethoxylated counterparts. In this sense, HPLC coupled with mass spectrometry (MS) including different ionization techniques and mass analysers has been shown to be very useful for the elucidation of porphyrin structures: laser desorption-TOF ([28], ESI-TOF [26], MALDI-TOF [29], APCI-TOF [11] and electron-induced dissociation (EID), collisionally activated dissociation (CAD) and infrared multiphoton dissociation (IRMPD) associated to Fourier transform ion cyclotron resonance (FTICR) [30]. The use of tandem mass spectrometry (MS/MS) to further distinguish structural configurations is reported infrequently and it is limited for specific chlorophyll derivatives: mainly chlorophyll *a* allomers [22-24, 31], native chlorophylls and pheophytins, pyro-pheophytins [32], and chlorophyll *b* allomers [21]. The absence of a complete analysis of the fragmentation pattern (MS^2) of the chlorophyll derivatives arising as consequence of the natural metabolism in photosynthetic organisms or during the food storage/processing is firstly due to the lack of specific protocols of standards production. On the other hand, is that the separation, MS^n analyses and data interpretation of chlorophyll standards is laborious and time consuming. Nowadays, MS methodologies have evolved positively, and allow measurement of exact mass and isotopic pattern by means of acquisition in high resolution mode. Only recently, high resolution and mass accuracy measurements have been applied successfully to MS analysis of one standard: chlorophyll *a* [30].

The application of software to assists in the predictions of fragmentation pattern, enhances the interpretation of intricate MS/MS spectra, allowing the analysis of a major number of standards under the same conditions.

In this study, new protocols for the preparation of the complete set of phytylated pyro-, 13^2-hydroxy- and 15^1-hydroxy-lactone chlorophyll standards are developed with the aim of obtaining high concentrated isolates for their subsequent MS^2 analyses. These were accomplished by the first time with the combination of high resolution time-of-flight (hrTOF) mass spectrometry and powerful post-processing software. The former allows the unequivocal measurement of the exact mass and isotopic pattern of chlorophyll derivatives and the latter can predict new product ions that were not described previously. As it has been proposed recently [30] new computational techniques are required to provide a complete understanding of the cleavage site and mechanisms channels after the MS^n fragmentation. Applications of this new strategy at once to the exhaustive and complete set of phytylated chlorophyll derivatives present *in vivo* in the vegetal kingdom, as well as the development of suitable preparation and isolation protocols have led to elucidate their complete fragmentation pathway, including new and exclusive fragmentations.

2. MATERIALS AND METHODS

2.1 Reagents.

Selenium dioxide (sublimed for synthesis, 98%) was supplied by Merck (Darmstadt, Germany). Ammonium acetate (technical grade, 98%), NaOH, NaCl were provided by Sigma-Aldrich (Steinheim, Switzerland). 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 solution of calibrant mix (ESI-L low concentration tuning mix, Agilent Technologies, Santa Clara, CA, USA) was used for MS calibration. Other reagents (pyridine, diethyl ether, acetone, HCl) were of analysis grade and supplied by Teknokroma (Barcelona, Spain).

2.2 Chlorophyll standards.

Chlorophyll *a* and *b* were respectively purchased from Wako (Neuss, Germany) and Sigma-Aldrich (Madrid, Spain) Chemical Co. Mg-free derivatives (pheophytin *a* and *b*) were obtained from their corresponding parent chlorophylls dissolved in diethyl ether by acidification with 2-3 drops of 5 M HCl [33]. The initial amount of parent chlorophyll (or pheophytin) for the substrate of

each one trial was fixed around 0.2 mg. The 13^2-hydroxy-chlorophyll *a/b* (or pheophytin *a/b*) derivatives were obtained by selenium dioxide (7mg/mL) oxidation of chlorophyll *a/b* (or pheophytin *a/b*) [34] in heated pyridine solution under argon. Heating conditions were verified from 60 ºC to 100 ºC and from 1h to 5h of reaction time. Two different protocols were assayed for the preparation of 15^1-hydroxy-lactone chlorophyll *a/b* (or pheophytin *a/b*). Firstly, it was obtained by alkaline oxidation of their parent chlorophylls or pheophytins in aqueous medium. For this purpose, solid and chromatographically pure chlorophyll and pheophytin (*a* or *b*) was dissolved in acetone and mixed with 0.5% NaOH and exposed to atmospheric oxygen at room temperature for 5-10 min. The resulting oxidation products were transferred to diethyl ether by addition of NaCl saturated water, and oxidative solutions were gathered and evaporated to dryness [27]. Another method used for the acquisition of 15^1-hydroxy-lactone derivatives was selenium dioxide oxidation of their parent chlorophylls or pheophytins (7mg/mL) at 70 ºC in pyridine solution under argon, from 3h to 9h. To produce pyro-derivatives, parent chlorophylls or pheophytins in pyridine solution blanketed with N_2 were directly heated at consistent temperature. Similarly, different heating temperatures ranging from 60ºC to 100 ºC and time (1h-8h) were executed to obtain the appropriate conditions.

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

The liquid chromatograph in the HPLC/APCI-hrTOF-MS system was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed as described previously [18] but using 1 M ammonium acetate in water as the ion reagent and 1.00 mL/min as flow rate. A split post-column of 0.25 mL/min was introduced directly on the mass spectrometer 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 APCI interface. The scan range applied was m/z 50-1500 and mass resolving power was always over 18.000 ($m/\Delta m$). 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 . All data were used to perform multitarget-screening using TargetAnalysis™ 1.2 software (Bruker Daltonics, Bremen, Germany). 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 Bruker Daltonics HyStar 3.2.

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 chlorophylls and their derivatives compounds. Data evaluation was performed with Bruker Daltonics DataAnalysis 4.0. From the HPLC/TOF-MS acquisition data, an automated peak detection on the EICs expected for the $[M+H]^+$ ions of each compound in the database was performed with Bruker Daltonics TargetAnalysis™ 1.2 software. The software performed the identification automatically according to mass accuracy and in combination with the isotopic pattern in the SigmaFit™ algorithm. This algorithm provides a numerical comparison of theoretical and measured isotopic patterns and can be utilized as an identification tool in addition to accurate mass determination. The calculation of SigmaFit values includes generation of the theoretical isotope pattern for the assumed protonated molecule and calculation of a match factor based on the deviations of the signal intensities. Only those hits with mass accuracy and SigmaFit values within the tolerance limits, which were set at 3 ppm and 50, respectively, are included in the final report list that was carried out using a Microsoft EXCEL-based script. The interpretation of the MS-MS spectra was performed using the SmartFormula3D™ module included in the DataAnalysis software. This module includes an algorithm that estimates whether a formula for a product ion is a subset of a formula for the precursor. Based on expected chemistry, elements carbon, hydrogen, oxygen, nitrogen, bromine and iodine were permitted. Sodium and potassium were also included for the calculation of adduct masses. The number of nitrogen atoms was limited to an upper threshold of ten. The number of rings plus double bonds was checked to be chemically meaningful (between 0 and 50). For each chlorophyll compound detected in the sample, the module shows the original MS and MS-MS data as peak lists. From all possible formulae for the precursor ion, only one should fit with the elemental composition expected for the $[M+H]^+$ ion and satisfy thresholds for mass accuracy and SigmaFit values. Once the correct formula is selected, the module displays the formulae and neutral losses in the MS-MS spectrum fitting to the boundary conditions for the precursor ion, and they should be consistent with the MS-MS data peak list. The SmartFormula3D checks the consistency highlighting the monoisotopic peaks with formula suggestion and the related isotopic peaks. Based on this combined data evaluation, fragmentation pattern for each chlorophyll compound can be generated to support its identification in the sample [35].

Mass Frontier™ 4.0 is a software package for the management, evaluation and interpretation of mass spectra, including the automated generation of possible product ions and rearrangement mechanisms, starting from a user-supplied chemical structure. With this feature of the software we can check consistency between a chemical structure and its mass spectrum and

recognize the structural differences between spectra of closely related compounds. The program generates a fragmentation scheme for the 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. The program parameters used in this study were APCI ionization method, inductive cleavage and 5 as maximum number of reaction steps. The fragmentation reactions were selected to include hetero and homolytic cleavage, neutral losses and hydrogen rearrangements. Other parameters were left as their default values.

3. RESULTS AND DISCUSSION

3.1 Preparation and isolation of chlorophyll standards.

Figure 1 shows the structures and numbering system of native chlorophylls *a* and *b*, and their phytylated oxidative derivatives identified in this work, which may occur *in vivo* in green tissues or in storage/processed foods. During the standards preparation and isolation, and the subsequent MS analysis, it was possible to obtain some chlorophyll derivatives that do not fulfill the MS criteria for compound identification, although they presented both UV-visible spectrum and retention time corresponding to the intended isolated standard. Some procedure protocols developed to date produce chlorophyll derivatives that are not present in vegetal tissues, while no specific methodology exists for the obtaining of some chlorophyll derivatives present *in vivo*.

As mentioned previously, the production and characterization of oxidized chlorophyll derivatives have been focused on allomerized chlorophylls, by the application of unrestricted oxidation of the chlorophyll solution in methanol with air, during hours to days [22-24, 26, 36]. This reaction produces mainly 15^1-methoxy-lactone and 13^2-methoxy chlorophylls while the 13^2-hydroxy chlorophyll derivatives account for a minor proportion of the total oxidized profile. Similar results are obtained with an alternative method that applies the alkaline oxidation (0.5% NaOH in methanol) during 5-10 min [7] to the chlorophyll solution. However, to the best of our knowledge, only one paper has described the specific production of 13^2-hydroxy chlorophyll *a*, by means of oxidation with SeO_2 in pyridine [34], but without any description of the reaction conditions (either temperature, nor reaction time or concentrations. In this reaction protocol the chlorophyll *a* substrate is diluted in pyridine so the source of methoxyl groups is avoided. For our purpose, it was necessary to fit the method for the production of all 13^2-hydroxy standards, keeping fixed the concentration of SeO_2, and the amount of the initial standard as low as possible (section 2.2), we verified temperatures from 60 to 100ºC from 1 to 5 hours. As shown in Table 1, the yields of 13^2-hydroxy derivatives were maxima at 70ºC 3 hours, decreasing with higher reaction times (5h), as

other side reactions progressed as well. At higher temperature (100ºC) only formed 13^2-hydroxy pheophytins in less amount, and any 13^2-hydroxy chlorophylls. Consequently, 70ºC and 3 hours were set up as the best conditions for 13^2-hydroxy formation.

Although 15^1-hydroxy-lactone derivatives are present regularly in vegetal tissues and foods, there is no method for the standard preparation. Consequently, we checked the consistency of two methods, alkaline oxidation and oxidation with SeO_2 (section 2.2). The 15^1-hydroxy-lactone chlorophyll derivatives present a higher oxidative stage than 13^2-hydroxy compounds [26]. Consequently the best experimental approach to obtain them, on the basis of the SeO_2 method, was to increase the oxidation time, starting from the best conditions applied for 13^2-hydroxy-standards (i.e. their substrates): 70ºC 3 hours and increasing the reaction time up to 9 hours. During this procedure, oxidation products produced at different time intervals were monitored and the results (data not shown) determined 7 hours as the best, decreasing the amounts of 15^1-lactone derivatives with more reaction time. The alternative method [27] for the preparation of 15^1-hydroxy-lactone chlorophyll was not suitable for these standards. Although some oxidation products with typical UV-vis spectra and chromatographically similar to 15^1-hydroxy-lactone chlorophyll standards were obtained, they do not fulfil the MS criteria and MS^2 fragmentation pattern of the intended standard, resembling the unspecific character of the allomerization reaction.

Classically, pyro-pheophytins *a* and *b* have been prepared by heating pheophytin solution in pyridine at temperatures higher than 100ºC during unspecific time (from 1 to 24 hours). To the best of our knowledge, only Pennington et al. [36] prepared pyro-chlorophyll under the same experimental conditions (100ºC), but starting from a highly concentrated solution and applying extensive heating time (50-100 mg; 24 hours). To validate the appropriate conditions the reaction was followed at 100ºC but reducing the initial amount of the standard (around 0.1 mg), as shown in Table 1. Although pyropheophytins are formed at high concentrations, pyro-chlorophyll derivatives are not obtained in reasonable amounts after heating a solution of native chlorophylls during 4 hours at 100ºC, probably due to their higher labile character. Consequently, we reduced heating temperature at from 60 to 80ºC at different times (1 to 8 hours), selecting 80ºC during 4 hours as the best conditions to produce 13^2-hydroxy standards, as enough amount of intended standard was obtained for MS analysis without requiring a highly concentrated starting solution. Consequently, our data showed that the presence of Mg^{2+} in the central position of the cyclic tetrapyrrol structure influenced the preparation procedures of other chlorophyll derivatives. Thus, the preparation of chlorophyll derivatives required lower heating temperatures than the corresponding related pheophytin derivatives, although Pennington et al. [36] reported the use of

the same temperature for both kinds of derivatives: chlorophylls and pheophytins. When starting from a determined initial concentration, positive results were obtained for Mg^{2+}-free derivatives, but not for the metal containing samples, which showed a different behaviour with temperature regimes, as well as the final intended standard which is more prone to oxidation in the case of Mg^{2+} containing derivatives. Several kinetic studies developed to measure the thermal stability of metal complexes show that the activation energy of the complex is relatively low, indicating the autocatalytic effect of the metal ion on the thermal decomposition of the complex [37] as overcome with a fine-tuning of the reaction conditions to heat-sensitive compounds or by increasing the substrate concentration, although as stated before, it is not convenient considering the limited availability of commercial chlorophyll standards especially for chlorophyll *b*.

3.2 Analysis of phytylated chlorophyll *a* and *b* derivatives by HPLC-APCI-hrTOF-MS^n.

Chlorophyll molecule presents two differentiated structural arrangements, the porphyrin ring and the phytol chain, while the chlorophyll derivatives analysed in this work are distinguished from the native compound in the oxygenated functions located at the isocyclic ring (R_2 and R_3 in Figure 1) and/or the co-ordinated metal atom that may be present or not (Figure 1). Combination of these structural modifications yields up to 14 chlorophyll derivatives so seven chlorophyll derivatives from each family (*a* and *b*) and the native compounds were analysed in this work by APCI 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 to the different product ions. These are two of the main benefits of the experimental design of this work, the acquisition of MS data in high resolution mode and the application of the same experimental conditions during the MS analyses to the 14 chlorophyll derivatives and their parent compounds. These advantages allow ascertaining accurately the nature of the product ions produced in the MS^2-based reactions for each standard, and a clear picture about the fragmentation pathways which are common or different in relation to the structural characteristics of the chlorophyll derivatives. Table 2 shows the molecular weight and elemental composition for the phytylated derivatives and MS criteria for compound identification, accurate mass and error (ppm), and the mSigma value obtained with the SigmaFit™ algorithm, an orthogonal criterion for compound identification independent of mass measurements, which indicates the agreement between the theoretical and the measured isotopic pattern of the mass signal of interest. The consistency of the elemental composition and formulae of all product ions was possible performing the APCI-hrTOF-MS^2 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).

The bbCID methodology allows to complete the evaluation of the MS^2-based reactions of the chlorophyll derivatives in addition to obtain the positively identification of the standard with the MS criteria (Table 2). The characteristic product ions of the matched compounds and their intensity (expressed in percentage term taking the intensity value of the most abundant product ion in each standard MS^2 analysis as 100) are shown in Table 3. Following the product ions on each case, it is possible to build the fragmentation pattern of the chlorophyll derivative and detect specific product ions that are singularly produced in a given compound as a consequence of its structural configuration. In this study we used predictive software for screening possible product ions which are generated *in silico* based on generally recognized fragmentation patterns and literature database. This feature of the new proposed method allows to identify easily not only product ions in the MS^2 analyses, but also new ones not previously recognized.

The typical ions derived from the fragmentation of native chlorophylls (which comprises chlorophyll *a* and *b* and their corresponding pheophytins) (compounds I, V, IX and XIII in Figure 2a) are those arising from the loss of the phytyl, the characteristic process for decomposition of esters that involves rearrangement of two hydrogen atoms (fragmentation C, Table 3). As the protonation site is closer to the propionic chain at C17, the phytyl chain fragmentation includes additional methylene groups and oxygen functions from C17, while oxygen functions from the $C13^2$ position are subsequently dissociated from the intermediate product ion (fragmentations E and F, Table 3) in agreement with previous literature [21, 24, 31-32], and this process takes place independently of the ionization technique applied. With a lesser extend some product ions were observed in specific standards. Thus, the native chlorophyll *a* and *b*, and pheophytin *a* and *b* present the complete loss of the phytyl chain with the propionic unit and the β-keto ester group from $C13^2$ (fragmentation G, Table 3) as well, a product ion with significant intensity values in the MS^2 spectra and characteristic of the native chlorophylls. It is important to highlight the product ions found exclusively in *b* series (chlorophyll and pheophytin *b*), arising from the cleavage of CO in combination with the loss of the phytyl (C+CO group, Table 3) or with the loss of the phytyl chain and partial fragmentation of the propionic unit (E+CO group, Table 3). In the identification of chlorophyll *b* allomers, Hyvärinen and Hynninen [21] found product ions which correspond with the E+CO fragment. Later, Gauthier-Jaques et al., [24] in the analysis of pheophytin *b* allomers identified a product ion at *m/z* 857 corresponding to $[M-28]^+$ and they proposed the fragmentation of the CO group from the aldehyde function at C7 as the most plausible description (Figure 3a, upper scheme), as only *b* derivatives posses a aldehyde function in C7 instead of the methyl

group in *a* series. However, another alternative is feasible. The proposal consists of the fragmentation of the keto group at C13^1 after ionization where occurs the inductive cleavage of the CO group (Figure 3a, lower scheme). This alternative does not implicate the aldehyde group at C7, but a new question arises, that is, the absence of CO fragmentation in MS^2 analyses of *a* series chlorophyll derivatives (with the exception of 15^1-hydroxy-lactone derivatives for the reason uncovered below) as the keto group at C13^1 is always present in any chlorophyll derivative. Although this alternative is also available for the chlorophyll derivatives of *a* series, our data confirm that only MS^2 analysis of chlorophyll derivatives from *b* series present product ions arising from fragmentation of a CO group. The elemental composition and formulae of these product ions were checked with the SmartFormula3D algorithm, obtaining positive records in all cases. In this case we should consider the stability of the product ion that arises from the reaction pattern at C13^1 (Figure 3b). In the case of chlorophyll derivatives of the *b* series the final product ion may stabilize by delocalization of the charge through the conjugated double bond system from the C13^1 to the aldehyde group at C7. Indeed, it is the presence of the oxygen function at that position that may contribute positively to stabilization of the product ion while this situation is not available in chlorophyll derivatives of *a* series.

With the exception of the CO fragmentation there is no other difference in the fragmentation profile of *a* and *b* series of native chlorophylls and pheophytins (see Table 3).

The 13^2-hydroxyl derivatives have been only extensively analysed independently, by MS^2, in the case of 13^2-hydroxy chlorophyll *a* [20, 22-23, 31], 13^2-hydroxy chlorophyll *b* [21] and 13^2-hydroxy pheophytin *a* [24]. In this work we performed the analysis of all these hydroxyl derivatives as well as the 13^2-hydroxy pheophytin *b* (not described so far). With the MS^2 analyses of the phytylated hydroxyl chlorophyll derivatives under the same experimental conditions (Figure 2b, Table 3) we can ascertain that all the 13^2-hydroxy derivatives follow the same MS^2-based reactions as their parent chlorophylls and pheophytins but include the dissociation of the hydroxyl group. Consequently, the main product ions are those arising from the loss of the phytyl chain (C´, Table 3) with some of the adjacent methylene groups and oxygen functions from C17 (E´, Table 3) and C13^2 (G´, Table 3), respectively. The presence of product ions retaining the hydroxyl function at C13^2 was very limited indicating that the loss involving that group occurs very easily. This is related with the stereochemical configuration of the hydroxyl group, which is exposed to steric repulsion when the C13^2 carbon-oxygen bond is in the same side of the macrocycle as the C17 phytyl and propionic chains (S configuration) [21, 23] increasing the ability of dissociation of the hydroxyl group. Data regarding the ratio among epimers after allomerization reaction of chlorophylls is limited to methoxy derivatives (as they are the major reaction products of this

reaction) and the S configuration is preferred but not significantly higher than the R epimer (57:43) [21]. It is possible that the modifications introduced in the original protocol, to increase the amount of hydroxyl-derivatives, may have modified the ratio among epimers raising the trend to S configuration what results in a minimal presence of product ions containing hydroxyl group at $C13^2$ in MS^2 analyses. In fact, there are MS^2 analysis in which the product ions retaining hydroxyl groups are the predominating ones [20-21] and *vice versa* [22,24]. The application of the post-processing software to MS^2 spectra for determining the structural configuration of the product ions has allowed the identification of some new ones in the case of hydroxyl chlorophyll derivatives. Thus, the signals at *m/z* 481.1848, 495.2209, 459.2169, 473.1954 (G´, Table 3) observed for 13^2-hydroxy derivatives corresponding to the loss of characteristic fragment ions [M-$C_{20}H_{38}$-CO_2CH_3-CH_2CO_2H-CH_3-OH]$^+$, and the signals at *m/z* 525.2132, 539.1901, 503.2195, 517.2196 (D´+B´, Table 3) also observed for 13^2-hydroxy derivatives, corresponding to the [M-$C_{20}H_{38}$-CO_2CH_3-CO_2-OH]$^+$ ion, are specific for hydroxyl chlorophyll derivatives and they have not been reported previously. The generation of product ions arising from fragmentations including a CO group (C´+CO group and E´+CO group, Table 3) are limited for *b* series of 13^2-hydroxyl chlorophyll derivatives in agreement with the MS^2 behaviour of their native substrates. It is interesting to note that the product ions noted as F´ in Table 3 are also limited to *a* series. Consequently, the introduction of the hydroxyl group at $C13^2$ modifies substantially the fragmentation pattern of phytylated chlorophyll derivatives, and such modification is affected by the substituent at C7.

The APCI-MS^2 spectra of 15^1-hydroxy-lactone derivatives have not been reported previously with the exception of 15^1-hydroxy-lactone chlorophyll *a* [31] accomplished by means of FAB-MS in that case. The presence of product ions with the hydroxyl group at $C15^1$ is minimal as described for the 13^2-hydroxy derivatives, and the same stereochemical effects caused by neighbouring groups (phytyl and propionic chains) participating in fragmentation reaction may happen. The major product ions (Figure 2c), considering their intensity values presented in Table 3, are those related with fragmentation of phytyl and propionic chains together with some of the adjacent oxygen functions at the isocyclic ring. An exception is the complete absence of the G fragmentation (which includes phytyl, and propionic chains and the ß-keto ester group) during the MS^2 analyses of the 15^1-hydroxy lactone derivatives (Table 3), which is always present in all the other chlorophyll derivatives. It seems that the lactone configuration inhibits the complete fragmentation. The most remarkable product ion of the 15^1-hydroxy-lactone chlorophyll derivatives exclusively detected in this set of chlorophyll standards, is the breakdown of the isocyclic ring (product ions noted as K and K´ in Table 3 and Figure 2c). Consistency of these

product ions, elemental composition and formulae, was checked with the SmartFormula3D algorithm obtaining positive results in all cases. To the best of our knowledge, the fragmentation of the isocyclic ring has been only proposed for 15^1-hydroxy-lactone chlorophyll *a* by Grese et al. [31] and they suggested the rearrangement of the complete functions at $C15^1$ and their subsequent dissociation from the main ion as the mechanism for the molecular ion fragmentation. In our fragmentation scheme, the ionization of the 15^1-hydroxy-lactone derivatives may take place at the double bond among C14 and 15 positions, then inductive cleavage of the C15-$C15^1$ bond and charge migration to the lactone group, and subsequent fragmentation of the hydroxyl and β-keto-ester functions. Another two exclusive fragmentations for 15^1-hydroxy-lactone chlorophyll derivatives, are the A´+D´ and the J´, (Table 3), but in this last case, they are exclusive for *a* series. The consistency of the product ions has been checked, and the exact mass and elemental composition matched with our requirements (section 2.4). Both fragmentations have not been reported previously although the *in silico* prediction performed with Mass Frontier™ points to such losses. Unexpectedly, the 15^1-hydroxy-lactone derivatives of the *b* series do not present the paired CO loss with any of the fragments involving phytyl and/or propionic chains observed in MS^2 analyses of the previous *b* series compounds (C+CO, C´+CO, E+CO or E´+EO). The absence of such fragmentations in 15^1-hydroxy-lactone derivatives MS^2 spectra is consistent with our proposal that the CO breakdown arises from the fragmentation of the keto group at $C13^1$ and not from the aldehyde group at C7 (Figure 3a). The reaction mechanism we propose (Figure 3b) is not possible for 15^1-hydroxy-lactone derivatives (either for *a* or *b* series) as the introduction of the lactone function draws the ionization to this group instead of to the keto function at $C13^1$.

The structural reduction attained in the native chlorophyll *a* and *b* to obtain their pyro-derivatives means a simplification of the APCI-MS^2 spectra of these compounds, which are frequently present in canned or heated green vegetable materials. The main product ions are those involving the fragmentation of the phytyl (C fragmentation) and propionic chains (D and F fragmentation) (Figure 2d, Table 3). This is in agreement with scarce related bibliography regarding the MS^2 analysis of pyro-pheophytin *a* and *b*, limited to the work published by van Breemen et al. [32] and the results reported by Gauthier-Jaques et al. [24] for pyro-pheophytin *a*. The product ions including dissociation of the CO group are limited to the *b* series of pyro-derivatives as observed in the parent compounds and 13^2-hydroxy derivatives (Table 3). An outstanding result is the fact-finding about the absence of product ions arising from E fragmentation in all the pyro-derivatives, which are relatively frequent and intense for the other chlorophyll derivatives. Lastly, pyro-derivatives present the product ions at *m/z* 467.2031, 481.2029, 445.2511, 459.2442, which have not been described to date corresponding with the

$[M\text{-}C_{20}H_{38}\text{-}CH_2CO_2CH_2\text{-}O]^+$ ion (F+O, Table 3). These product ions are derived from the charge-site rearrangement and inductive cleavage that leads to the fragmentation of the phytyl and propionic chains at C17, and the subsequent charge remote rearrangement and dissociation of the keto function at C13[1]. The study of all the phytylated pyro-chlorophyll derivatives allows us to conclude that the presence/absence of coordination with Mg^{+2} does not affect the MS^2 fragmentation pattern of pyro-derivatives.

4. CONCLUSIONS

It must be highlighted that there are characteristic fragmentations recurrently present in all the MS^2 analyses of phytylated chlorophylls, independently of the substitutes of the porphyrin ring. Such fragmentations correspond to the phytyl chain fragmentation and progressive breakdowns from the propionic chain at C17 (excluding the pyro-derivatives). The introduction of functional groups at the isocyclic ring (hydroxyl, hydroxyl plus lactone or the pyro- rearrangement) of the chlorophyll molecule implies mainly the presence of such characteristic product ions and the appearance of some new ones arising from fragmentations and consecutive reactions in the isocyclic ring. In fact, such modifications in the isocyclic ring increase the differences in the MS^2 fragmentation profile among *a* and *b* series, yielding new and specific product ions. On the other hand, the central coordination of the macrocycle with magnesium (chlorophylls) or with hydrogen atoms (pheophytins) does not alter the fragmentation pattern. Such conclusions have been obtained thanks to the new methodology proposed, which includes the high resolution and the study of the results with powerful post-processing software. The use of new computational techniques are essential to provide a complete understanding of the cleavage site and mechanisms channels after the MS fragmentation, as it has been shown for phytylated chlorophylls. The application of new methods of analysis able to determine new product ions is essential for estimation of natural components in living organisms, as they can be unique probes for adulteration control [37] and metabolomics [38].

ACKNOWLEDGEMENTS

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REFERENCES

[1] G. D. Díaz, Q. Li, R. H. Dashwood, Caspase-8 and apoptosis-inducing factor mediate a cytochrome c-independent pathway of apoptosis in human colon cancer cells induced by the dietary phytochemical chlorophyllin, Cancer Res. 63 (2003) 1254-61.

[2] M. T. Simonich, P. A. Egner, B. D. Roebuck, G. A. Orner, C. Jubert, C. Pereira, J. D. Groopman, T. W. Kensler, R. H. Dashwood, D. E. Williams, G. S. Bailey, Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat, Carcinogenesis 28 (2007) 1294-1302.

[3] T. Negishi, H. Rai, H. Hayatsu, Antigenotoxic activity of natural chlorophylls, Mutat. Res. 376 (1997) 97-100.

[4] U. M. Lanfer-Marquez, R. M. C. Barros, P. Sinnecker, Antioxidant activity of chlorophylls and their derivatives, Food Res. Int. 38 (2005) 885-91.

[5] S. Schelbert, S. Aubry, B. Burla, B. Agne, F. Kessler, K. Krupinska, S. Hörtensteiner, Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis, Plant Cell 21 (2009) 767–785.

[6] N. Turkmen, E. S. Poyrazoglu, F. Sari, Y. S. Velioglu, Effects of cooking methods on chlorophylls, pheophytins and colour of selected green vegetables, Int. J. Food Sci. Technol. 41 (2006) 281–288. doi: 10.1111/j.1365-2621.2005.01061.x.

[7] R. Sanchez-Vega, P. Elez-Martinez, O. Martin-Belloso, O. Martin-Belloso, Effects of High-Intensity Pulsed Electric Fields Processing Parameters on the Chlorophyll Content and Its Degradation Compounds in Broccoli Juice, Food Bioprocess Technol. 7 (2014) 1137-1148. doi: 10.1007/s11947-013-1152-2

[8] F. L. Canjura, S. J. Schwartz, Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chromatography. J. Agric. Food Chem. 39 (1991) 1102–1105.

[9] M. I. Mínguez-Mosquera, J. Garrido-Fernández, Chlorophyll and carotenoid presence in olive fruit (Olea europaea, L.). J. Agric. Food Chem. 37 (1989) 1-7.

[10] L. Gallardo-Gerrero, B. Gandul-Rojas, M. I. Míguez-Mosquera, Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (pisum sativum L.), J. Agric. Food. Chem. 56 (2008) 8379–8386.

[11] S. C. Huang, C. F. Hung, W. B. Wu, B.H. Chen, Determination of chlorophylls and their derivatives in Gynostemma pentaphyllum Makino by liquid chromatography-mass spectrometry, J. Pharm. Biomed. Anal. 48 (2008) 105-12.

[12] C.H. Loh, B.S. Inbaraj, M.H. Liu, B.H. Chen, Determination of chlorophylls in Taraxacum formosanum by high-performance liquid chromatography-diode array detection-mass

spectrometry and preparation by column chromatography, J. Agric. Food Chem. 60 (2012) 6108-15.

[13] L. Gallardo-Guerrero, B. Gandul-Rojas, M. Roca, M. I. Mínguez- Mosquera, Effect of storage on the original pigment profile of Spanish virgin olive oil. J. Am. Oil Chem. Soc. 82 (2005) 33-39 doi:10.1007/s11746-005-1039-1048.

[14] N. Yamauchi, K. Eguchi, In vitro chlorophyll degradation involved in flavonoid radical formed by chlorophyll-degrading peroxidase in flavedo extract of citrus nagatoyuzukichi fruit, J. Jpn. Soc. Hortic. Sci. 71 (2002) 243–248.

[15] Y. Funamoto, N. Yamauchi, M. Shigyo, Involvement of peroxidase in chlorophyll degradation in stored broccoli (Brassica oleracea L.) and inhibition of the activity by heat treatment, Postharvest Biol. Technol. 28 (2003) 39–46.

[16] T. H. Kao, C. J. Chen, B.H. Chen, An improved high performance liquid chromatography-photodiode array detection-atmospheric pressure chemical ionization-mass spectrometry method for determination of chlorophylls and their derivatives in freeze-dried and hot-air-dried Rhinacanthus nasutus (L.) Kurz, Talanta 86 (2011) 349-355.

[17] A. Otsuki, M. M. Watanabe, K. Sugahara, Chlorophyll pigments in methanol extracts from ten axenic cultured diatoms and three green algae as determined by reverse phase hplc with fluorometric detection, , J. Phycol. 23 (1987) 406–414.

[18] M. Roca, B. Gandul-Rojas, M. I. Minguez-Mosquera, Varietal differences in catabolic intermediates of chlorophylls in Olea europaea (L.) fruit cvs. Arbequina and Blanqueta, Postharvest Biol. Technol. 44 (2007) 150–156.

[19] R. Willstätter, A. Stoll, Untersuchungen über Chlorophyll: Methoden und Ergebnisse,. Julius Springer-Verlag, Berlin (1913) 53–125.

[20] R. Kostiainen, K. Hyvärinen, P. H. Hynninen, Fast-atom bombardment mass spectra of the 132-epimers of 132-hydroxychlorophyll a, Rapid Commun. Mass Spectrom. 9 (1995) 555-558. doi: 10.1002/rcm.1290090702

[21] K. Hyvärinen, P. H. Hynninen, Liquid chromatographic separation and mass spectrometric identification of chlorophyll b allomers, J. Chromatogr. A. 837 (1999) 107–116.

[22] C. Jie, J. S. Walker, B. J. Keely, Atmospheric pressure chemical ionisation normal phase liquid chromatography mass spectrometry and tandem mass spectrometry of chlorophyll a allomers, Rapid Commun. Mass Spectrom. 16 (2002) 473-479.

[23] J. S. Walker, C. Jie, B.J. Keely, Identification of diastereomeric chlorophyll allomers by atmospheric pressure chemical ionisation liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 1125-31.

[24] A. Gauthier-Jaques, K. Bortlik, J. Hau, L.B. Fay, Improved method to track chlorophyll degradation, J. Agric. Food Chem. 49 (2001) 1117-22.

[25] P. Kuronen, K. Hyvärinen, P. H. Hynninen, High-performance liquid chromatographic separation and isolation of the methanolic allomerization products of chlorophyll a, J. Chromatogr. A. 654 (1993) 93-104.

[26] P. H. Hynninen, K. Hyvärinen, Tracing the allomerization pathways of chlorophylls by 18O-labeling and mass spectrometry, J. Org. Chem. 67 (2002) 4055-4061.

[27] M. I. Mínguez-Mosquera, B. Gandul-Rojas, High-performance liquid chromatographic study of alkaline treatment of chlorophyll, J. Chromatogr. A. 690 (1995) 161-176. doi:10.1016/0021-9673(94)00924-X

[28] J. C. Tabet, M. Jablonski, R. J. Cotter, J. E. Hunt, Time-resolved laser desorption. III. The metastable decomposition of chlorophyll-a and some derivatives, Int. J. Mass Spectrom. Ion Processes 65 (1985) 105-117.

[29] T. Suzuki, H. Midonoya, Y. Shioi, Analysis of chlorophylls and their derivatives by matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry. Anal Biochem 390 (2009) 57−62. doi:10.1016/j.ab.2009.04.005.

[30] J. Wei, H. Li, M. P. Barrow, P. B. O'Connor, Structural Characterization of Chlorophyll-a by High Resolution Tandem Mass Spectrometry, J. Am. Soc. Mass Spectrom 24 (2013) 753-760. doi: 10.1007/s13361-013-0577-1

[31] R. P. Grese, R. L. Cerny, M. L. Gross, M. Senge, Determination of structure and properties of modified chlorophylls by using fast atom bombardment combined with tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 1 (1990) 72-84. doi:10.1016/1044-0305(90)80008-B.

[32] R. B. Van Breenmen, F. L. Canjura, S.J. Schwartz, Identification of chlorophyll derivatives by mass spectrometry, J. Agric. Food Chem. 39 (1991) 1452-1456.

[33] G. Sievers, P. Hynninen, Thin-layer chromatography of chlorophylls and their derivatives on cellulose layer, J. Chromatograph. 134 (1977) 359-364.

[34] T. Laitalainen, J. Pitkanen, P. Hynninen, Diastereoselective 132-hydroxylation of chlorophyll a with SeO2, in: IUPAC (Eds.), Abstracts of the 8th International IUPAC Conference on Organic Synthesis, Helsinki, 1990, pp.246.

[35] J. J. Ríos, A. Pérez-Gálvez, M. Roca, Non-fluorescent chlorophyll catabolites in quince fruits, Food Res. Int. 65 (2014) 255–262. doi: 10.1016/j.foodres.2014.03.063.

[36] F. C. Pennington, H. H. Strain, W. A. Svec, J. J. Katz, Preparation and properties of pyrochlorophyll a, methyl pyrochlorophyllide a, pyropheophytin a, and methyl pyropheophorbide a derived from chlorophyll by decarbomethoxylation, J. Am. Chem. Soc. 86 (1964) 1418-1426.

[37] A. M. El-Awad, Catalytic Effect of Some Chromites on the Thermal Decomposition of KClO4. Mechanistic and non-isothermal kinetic studies, J. Therm. Anal. Calorim. 61 (2000) 197-208. doi: 10.1023/A:1010137313622

[38] A. Pérez-Gálvez, M. Roca, A new probe for tracking the presence of E141i colorant in food. Food Control, 51 (2015) 240-243. doi: 10.1016/j.foodcont.2014.11.028.

[39] J. J. Ríos, M. Roca, A. Pérez-Gálvez A. Systematic HPLC/ESI-high resolution-qTOF-MS methodology for catabolomic studies in nonfluorescent chlorophyll catabolites pathway. J. Anal. Methods Chem. 2015 (2015). doi: 10.1155/2015 490627.

FIGURE LEGENDS

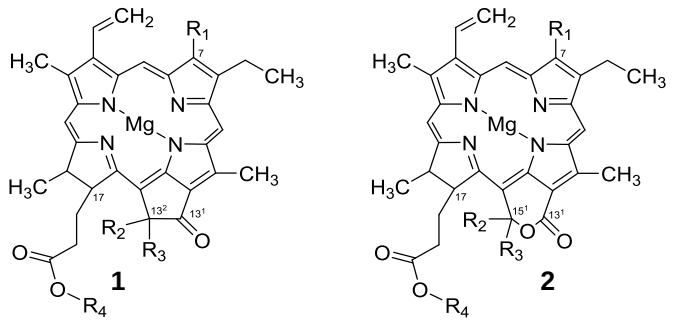
Figure 1.- Structural configuration names and numbering system for parent chlorophyll *a* and *b*, and their phytylated derivatives originated by different protocols and analysed by HPLC-APCI-hrTOF-MS.

Figure 2.- Sites of fragmentation occurring during HPLC-APCI-hrTOF-MS^2 analysis of phytylated 13^2-hydroxy, 15^1-hydroxy-lactone, and pyro- derivatives (**b**, **c**, **c**, respectively) and their parent chlorophylls (**a**) designed following the labelling discussed by van Breemen et al., (1991). Fragments including hydroxyl group from $C13^2$ are noted with apostrophe mark. For structural identification of radicals R_1 to R_4 and numbering system see Figure 1.

Figure 3.- Alternatives for ionization (at C7 or at $C13^1$) and subsequent fragmentation of CO group occurring in all *b* chlorophyll derivatives with the exception of 15^1-hydroxy-lactone (**a**). Stabilization of the product ion through the double bond conjugated system from C7 to $C13^1$ enhanced in *b* series if the ionization occurs in $C13^1$ (**b**).

Figure 1

FIGURE 1



Compound	Structure	Mg[a]	R_1	R_2	R_3	R_4
Chlorophyll *a*	1	+	CH_3	H	$COOCH_3$	phytyl
13^2-Hydroxy-chlorophyll *a*	1	+	CH_3	OH	$COOCH_3$	phytyl
15^1-Hydroxy-lactone chlorophyll *a*	2	+	CH_3	OH	$(15^2)COOCH_3$	phytyl
Pyro-chlorophyll *a*	1	+	CH_3	H	H	phytyl
Chlorophyll *b*	1	+	CHO	H	$COOCH_3$	phytyl
13^2-Hydroxy chlorophyll *b*	1	+	CHO	OH	$COOCH_3$	phytyl
15^1-Hydroxy-lactone chlorophyll *b*	2	+	CHO	OH	$(15^2)COOCH_3$	phytyl
Pyro-chlorophyll *b*	1	+	CHO	H	H	phytyl
Pheophytin *a*	1	-	CH_3	H	$COOCH_3$	phytyl
13^2-Hydroxy pheophytin *a*	1	-	CH_3	OH	$COOCH_3$	phytyl
15^1-Hydroxy-lactone pheophytin *a*	2	-	CH_3	OH	$(15^2)COOCH_3$	phytyl
Pyro-pheophytin *a*	1	-	CH_3	H	H	phytyl
Pheophytin *b*	1	-	CHO	H	$COOCH_3$	phytyl
13^2-Hydroxy pheophytin *b*	1	-	CHO	OH	$COOCH_3$	phytyl
15^1-Hydroxy-lactone pheophytin *b*	2	-	CHO	OH	$(15^2)COOCH_3$	phytyl
Pyro-pheophytin *b*	1	-	CHO	H	H	phytyl

[a]for pheophytin derivatives Mg atom is substituted by two hydrogen atoms

Figure 2

FIGURE 2

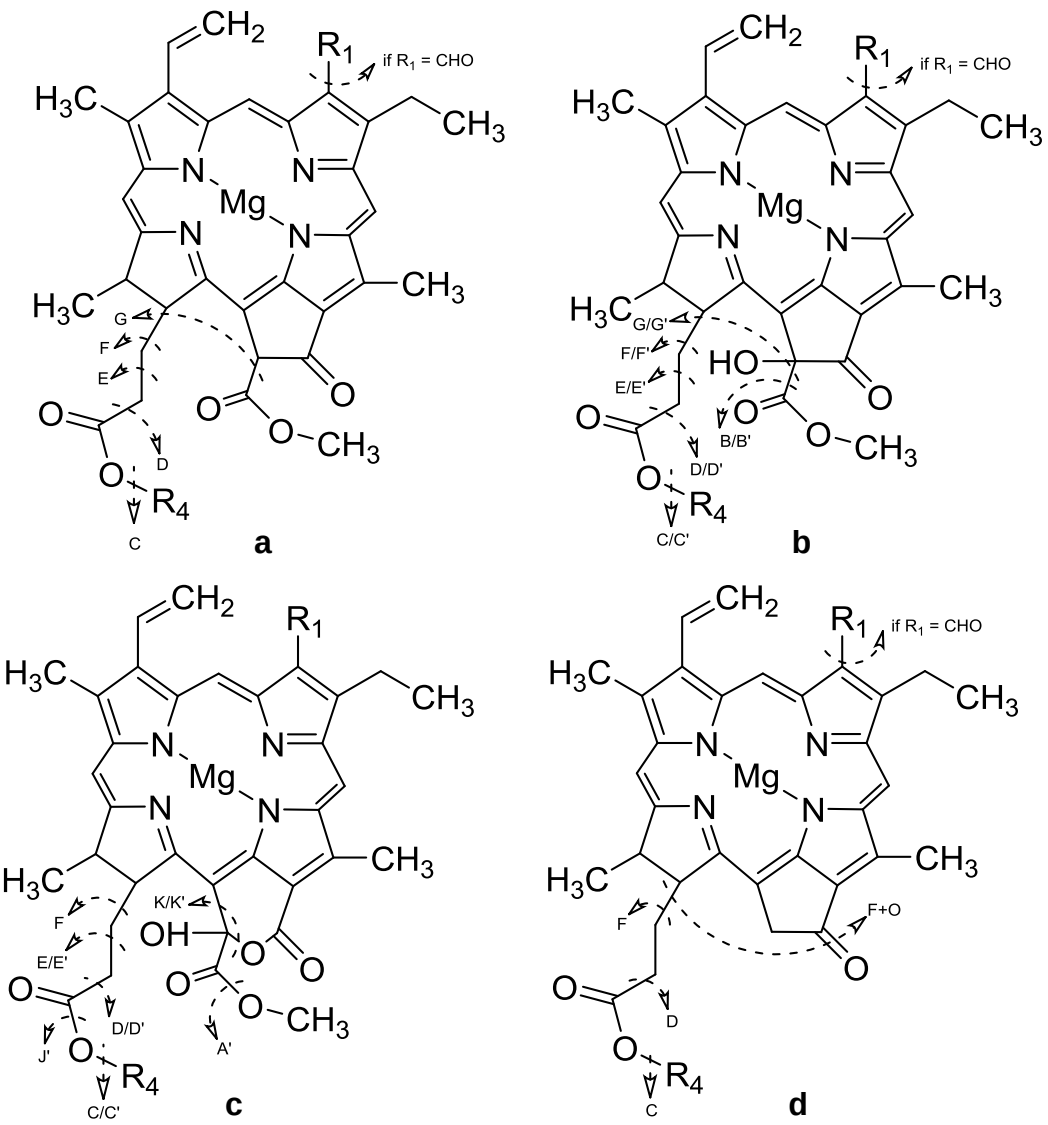
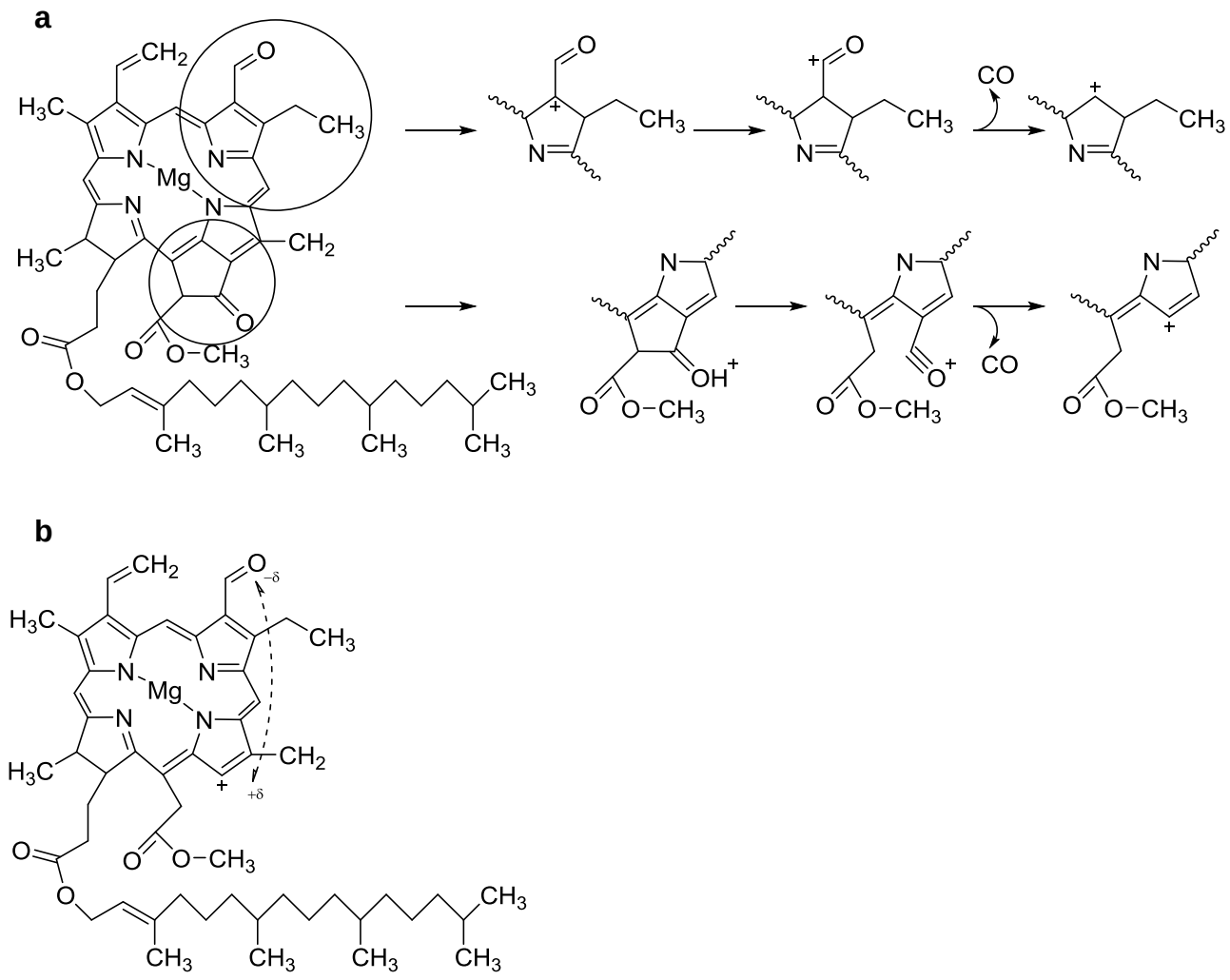


FIGURE 3



Tables

Table 1.- Conditions applied for the preparation of chlorophyll derivatives standards and subsequent analysis by HPLC-APCI-hrTOF-MS results.

	13^2-Hydroxy-derivatives			15^1-Hydroxy-lactone-derivatives		Pyro-derivatives			
	70ºC 1 h	70ºC 3 h	100ºC 1 h	0.5% NaOH	SeO_2 oxidation	60ºC 8h	70ºC 5h	80ºC 4h	100ºC 4h
Chlorophyll *a*	-	+	-	-	+	-	-	+	-
Chlorophyll *b*	-	+	-	-	+	-	-	+	-
Pheophytin *a*	-	+	+	-	+	n.d.	n.d.	+	+
Pheophytin *b*	-	+	+	-	+	n.d.	n.d.	+	+

Pyro-derivatives were isolated from a heated chlorophyll/pheophytin solution in pyridine.
13^2-hydroxy-derivatives were isolated from a heated chlorophyll/pheophytin solution containing 0.75 mg/mL SeO_2.
15^2-hydroxy-lactone-derivatives were isolated from a chlorophyll/pheophytin solution oxidized with 0.5% NaOH in an open vessel for 5-10 min or heating at 70 ºC for 7 h with SeO_2.
$^{-}$, $^{+}$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< 3 ppm, SigmaFit< 50).
n.d. not determined.

Table 2.- Retention time and high accuracy measurements of phytylated chlorophyll standards derivatives analysed by APCI-hrTOF-MS.

Compound	t_R (min)	Error (ppm)	mSigma	Molecular formula	$[M+H]^+$ (*m/z*)	
					MW. calc.	MW. meas.
Chlorophyll *a*	21.5	-0.3	35.4	$C_{55}H_{72}MgN_4O_5$	893.5426	893.54.29
13^2-Hydroxy-chlorophyll *a*	18.0	-0.2	19.3	$C_{55}H_{72}MgN_4O_6$	909.5375	909.5373
15^1-Hydroxy-lactone chlorophyll *a*	16.5	1.4	4.9	$C_{55}H_{72}MgN_4O_7$	925.5324	925.5338
Pyro-chlorophyll *a*	21.0	1.7	26.6	$C_{53}H_{70}MgN_4O_3$	835.5371	835.5357
Chlorophyll *b*	17.1	0.3	15.2	$C_{55}H_{70}MgN_4O_6$	907.5219	907.5216
13^2-Hydroxy-chlorophyll *b*	15.6	-0.4	8.7	$C_{55}H_{70}MgN_4O_7$	923.5168	923.5161
15^1-Hydroxy-lactone chlorophyll *b*	13.9	0.3	23.7	$C_{55}H_{70}MgN_4O_8$	939.5117	939.5120
Pyro-chlorophyll *b*	18.6	-2.9	24.0	$C_{53}H_{68}MgN_4O_4$	849.5164	849.5189
Pheophytin *a*	27.5	-1.3	19.7	$C_{55}H_{74}N_4O_5$	871.5732	871.5744
13^2-Hydroxy-pheophytin *a*	26.0	-0.6	44.5	$C_{55}H_{74}N_4O_6$	887.5681	887.5686
15^1-Hydroxy-lactone pheophytin *a*	24.2	-1.7	38.1	$C_{55}H_{74}N_4O_7$	903.5630	903.5638
Pyro-pheophytin *a*	31.2	0.1	11.2	$C_{53}H_{72}N_4O_3$	813.5677	813.5676
Pheophytin *b*	25.7	2.1	6.5	$C_{55}H_{72}N_4O_6$	885.5525	885.5543
13^2-Hydroxy-pheophytin *b*	19.2	0.7	37.1	$C_{55}H_{72}N_4O_7$	901.5474	901.5470
15^1-Hydroxy-lactone pheophytin *b*	19.6	3.0	38.0	$C_{55}H_{72}N_4O_8$	917.5423	917.5392
Pyro-pheophytin *b*	28.4	-0.2	7.3	$C_{53}H_{70}N_4O_4$	827.5470	827.5468

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 (<±3 ppm and <50, respectively).

Table 3.- Product ions (*m/z*) in the APCI-hrTOF-MS^2 spectra of phytylated chlorophyll derivative standards.

Compound[a]	C[b]	C'	C+CO	C'+CO	C'+14	D	E	E'	E+CO	E'+CO	F	F'	F+O	G	G'	J'	K	K'	A'+ D'	D'+ B'
I	615,2576 (12)[c]						555,2252 (100)				541,2058 (6)			481,1854 (17)						
II	631,2420 (1)	614,2434 (14)				585,2065 (1)	571,2191 (3)	555,2224 (100)			557,2165 (8)	541,2105 (3)		497,2179 (1)	481,1848 (19)					525,2132 (1)
III	647,2367 (1)	631,2327 (8)				601,2231 (7)	587,2463 (1)	541,2165 (64)			573,2220 (6)					615,2279 (20)	837,5211 (1)	821,5212 (1)	553,2073 (100)	
IV	557,2383 (100)					511,2641 (6)					483,2001 (12)		467,2031 (2)							
V	629,2156 (15)		601,2414 (9)			583,2089 (1)	569,2015 (100)		541,2086 (36)		555,2062 (2)			495,1692 (17)						
VI	645,2158 (53)	629,2196 (34)		601,2141 (12)		599,2010 (44)	585,1924 (96)	569,1890 (100)		541,1974 (45)	571,2209 (22)				495,2209 (19)					539,1901 (72)
VII	661,2328 (2)	645,2117 (29)				615,2024 (20)	601,2091 (17)	585,1902 (100)			587,1994 (21)					629,2098 (57)	851,5107 (2)		584,2019 (37)	
VIII	571,2188 (100)		543,2202 (60)			525,2161 (23)					497,1858 (15)		481,2029 (1)							
IX	593,2751 (100)					547,2684 (1)	533,2535 (76)				519,2421 (4)			459,2171 (11)						
X	609,2738 (1)	593,2759 (100)				563,2419 (1)	549,2518 (1)	533,2555 (67)			535,2614 (8)	519,2376 (6)		475,2316 (1)	459,2169 (11)					503,2195 (1)
XI	625,2708 (8)	609,2647 (87)			593,2598 (27)	579,2413 (11)	565,2443 (11)	549,2506 (100)			554,2644 (21)						815,5316 (3)		548,2359 (23)	
XII	535,2706 (100)					489,2666 (3)					461,2317 (21)		445,2511 (18)							
XIII	607,2549 (44)		579,2595 (18)			561,2469 (1)	547,2333 (100)		519,2368 (8)		533,2155 (4)			473,1959 (6)						
XIV		607,2540 (100)		579,2672 (31)		577,2445 (9)		547,2363 (89)		519,2233 (11)	549,2413 (26)				473,1954 (4)					517.2196 (9)
XV					607,2620 (100)	593,2557 (29)	579,2660 (77)				568,1836 (28)						829,5407 (26)		562,2188 (39)	
XVI	549,2487 (100)		521.2536 (54)			503,2468 (7)					475,2134 (53)		459,2442 (4)							

[a], I: chlorophyll *a*; II: 13^2-hydroxy-chlorophyll *a*; III: 15^1-hydroxy-lactone-chlorophyll *a*; IV: pyro-chlorophyll *a*; V: chlorophyll *b*; VI: 13^2-hydroxy-chlorophyll *b*; VII: 15^1-hydroxy-lactone-chlorophyll *b*; VIII: pyro-chlorophyll *b*; IX: pheophytin *a*; X: 13^2-hydroxy-pheophytin *a*; XI: 15^1-hydroxy-lactone-pheophytin *a*; XII: pyro-pheophytin *a*; XIII: pheophytin *b*; XIV: 13^2-hydroxy-pheophytin *b*; XV: 15^1-hydroxy-lactone-pheophytin *b*; XVI: pyro-pheophytin *b*.

[b], for fragment identification see Figure 2. Apostrophe mark indicates that the 13^2 hydroxy group is also fragmented.

[c] Relatives intensities for each *m/z* value appear in parentheses and are expressed as a percentage of the most abundant fragment ion in each standard.