Identification of Chlorophyll Derivatives by Mass Spectrometry†

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Chlorophylls *a* and *b* and the corresponding pheophytins, chlorophyllides, pheophorbides, and pyropheophytins were extracted from fresh spinach leaves or heat-treated spinach tissue, and zinc pheophytin *a* and zinc pyropheophytin *a* were prepared from pheophytin *a* and pyropheophytin *a*, respectively. Following separation using either reversed- or normal-phase HPLC, the molecular weight of each chlorophyll derivative was confirmed by positive ion fast atom bombardment (FAB) mass spectrometry. The isotope pattern of each molecular ion indicated whether or not the chlorophyll derivative contained magnesium or zinc. Next, tandem mass spectrometry (MS/MS) following collisional activation was used to obtain structural information that characterized each class of chlorophyll with respect to the presence or absence of the phytyl chain and the β-keto ester group on C-10. These HPLC and mass spectrometric data will serve as useful references for the isolation and structural determination of chlorophyll derivatives from synthetic or natural sources.

INTRODUCTION

Chlorophyll pigments have been the topic of enormous research efforts because of their prominent function in photosynthesis and plant physiology. Investigations spanning almost 100 years have led to the complete structural elucidation of chlorophyll (Goodwin, 1976). Despite these efforts, questions remain concerning the biosynthesis of these photosynthetic pigments. In particulr, the biochemistry involved in ripening as well as the catabolism of chlorophyll during senescence in plant tissues remains a biological enigma (Gross, 1987).

In early studies, chlorophyll appeared to decompose without the appearance of visible intermediate degradation compounds. It has been generally thought that decomposition occurs via release of chlorophyll from its protein complex followed by dephytylization catalyzed by the enzyme chlorophyllase and possibly pheophytinization via loss of the Mg^{2+} ion. Photooxidation of the porphyrin ring structure ensues, ultimately leading to the formation of colorless end products. The question of reutilization of the end products remains unsolved. In addition, the formation of the chlorophyll derivatives in edible plant tissues is an important factor in the maintenance of food quality (Schwartz and Lorenzo, 1990). For example, the use of zinc salts to form Zn–pheophytin complexes and stabilize color in thermally processed vegetables is currently being considered (LaBorde and von Elbe, 1990).

In part, previous studies have been hampered by the lack of sensitive detection techniques for the analysis and confirmation of derivatives which arise during metabolism, senescence, and processing of food products. In this study, we report the mass spectra of chlorophylls *a* and *b* and 10 of their derivatives including two zinc-containing derivatives.

Mass spectra of chlorophyll *a* (**1a**) (Figure 1) have been obtained by using desorption ionization techniques including laser desorption (Posthumus et al., 1978; Tabet et al., 1985; Grotemeyer et al., 1986), field desorption (Dougherty et al., 1980), plasma desorption (Hunt et al., 1981; Chait and Field, 1984), fast atom bombardment (FAB) (Barber et al., 1982; Brereton et al., 1983), and "in beam" (desorption) electron impact ionization (Constantin et al., 1981). FAB ionization combined with collisional activation and tandem mass spectrometry (MS/MS) have also been

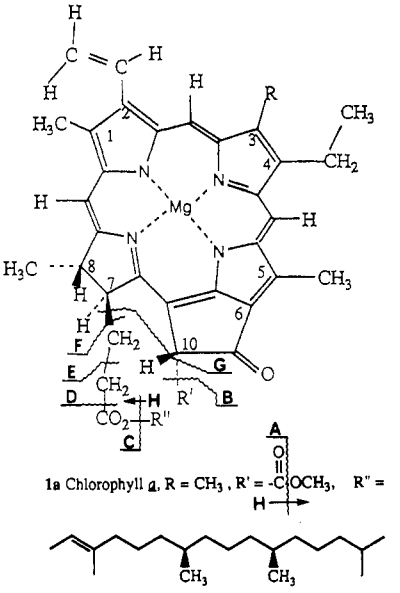

Figure 1. Structures of chlorophylls (**1**), pheophytins (**2**), chlorophyllides (**3**), pheophorbides (**4**), pyropheophytins (**5**), zinc pheophytin *a* (**6**), and zinc pyropheophytin *a* (**7**). Sites of fragmentation common to the chlorophyll derivatives are labeled A, B, C, etc. and are discussed in the text. Hydrogen transfers occurring during fragmentation are indicated by H →.

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applied to the analysis of chlorophyll *a* (Bricker and Russell, 1986), pheophytin *a* (**2a**) (Figure 1), bacteriochlorophyll *a*, and several monochlorinated and hydroxylated chlorophyll derivatives (Grese et al., 1990). Ionization by field desorption followed by MS/MS analysis has also been used to obtain structurally significant fragment ions of pheophytin *a* (Jackson, 1979).

In this investigation, FAB ionization combined with collisional activation and MS/MS analysis were carried out to confirm the structures of a related series of chlorophyll derivatives obtained by HPLC separation (Canjura and Schwartz, 1991) of a spinach extract. The compounds included chlorophylls *a* and *b* (**1**), the corresponding pheophytins (**2**), chlorophyllides (**3**), pheophorbides (**4**), and pyropheophytins (**5**), and the zinc-containing derivatives zinc pheophytin *a* (**6**) and zinc pyropheophytin *a* (**7**) (Figure 1).

EXPERIMENTAL PROCEDURES

Fresh spinach (100 g) from local markets was washed, drained, chopped (approximately 1 cm^2), and blended with 100 g of water for 4 min to obtain a homogeneous puree. The puree was degassed under reduced pressure. Chlorophylls *a* and *b* were extracted from 5.0 g of fresh spinach puree with 19 mL of acetone by using a Tissumizer Model TR-10Z (Tekmar Co., Cincinnati, OH) for 2 min, filtered through Whatman No. 1 and then Whatman No. 42 filter paper, and brought to volume with acetone/water (80/20) in a 25-mL volumetric flask.

Pheophytins *a* and *b* were prepared from the chlorophyll extract by acidification with 1.0 M HCl added dropwise to 15 mL. Conversion was reached in approximately 10 min and was verified by using HPLC with diode array absorbance detection (Canjura and Schwartz, 1991). Diethyl ether (10 mL) was added, and the solution was washed four times with water (5 mL). The organic layer was dried over anhydrous sodium sulfate and then evaporated under nitrogen. The pheophytins were then dissolved in acetone. Pyropheophytins were formed from heat-processed spinach puree (121 °C for 1 h) as previously described (Canjura et al., 1991).

Chlorophyllides were formed from fresh spinach leaves by incubation at 65 °C for 30 min to activate the enzyme chlorophyllase. The chlorophyllides were then extracted with acetone (Canjura and Schwartz, 1991). Pheophorbides were prepared from the chlorophyllide extract by acidification with HCl according to the procedure described above for the formation of pheophytins.

Zinc pheophytin *a* and zinc pyropheophytin *a* were formed from pheophytin and pyropheophytin extracts, respectively. Zinc chloride (0.3 g) was added to 4.0 mL of the pheophytin and/or pyropheophytin extract as previously described by Schwartz (1984). After completion of the reaction, the pigments were transferred into hexane for analysis.

The HPLC procedures previously described by Canjura and Schwartz (1991) were used to resolve the chlorophyll components. Each pigment derivative was collected as it eluted from the chromatographic column and saved for analysis by mass spectrometry. Water was eliminated from the collected mixture by extracting the pigments with diethyl ether and concentrating under nitrogen. Chlorophylls and derivatives were dissolved in diethyl ether or hexane, and chlorophyllides and pheophorbides were freeze-dried and then dissolved in acetone. All handling procedures were carried out under subdued light.

Positive ion FAB mass spectra were obtained by using a JEOL (Tokyo) JMS-HX110HF double-focusing mass spectrometer equipped with a JMA-DA5000 data system, collision cell in the first field-free region, and B/E linked scanning. The accelerating voltage was 10 keV, and the resolving power was 1000 for all measurements. During the acquisition of each mass spectrum, 7–10 scans were acquired and averaged by the data system. Approximately 1 μg of each sample, dissolved in acetone, was added to 1 μL of the FAB matrix, 3-nitrobenzyl alcohol, and analyzed by positive ion FAB mass spectrometry.

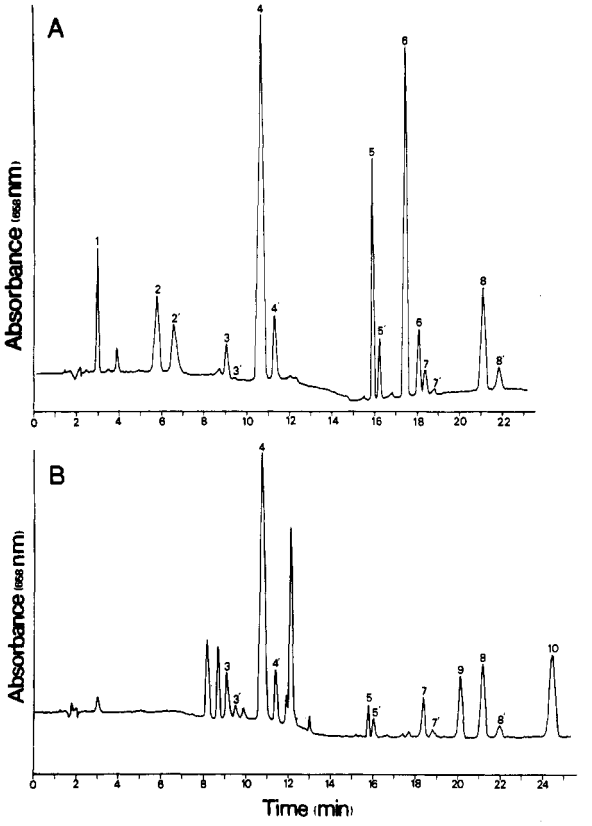

Figure 2. HPLC chromatograms of spinach extracts: (A) incubated 65 °C (30 min) and heated 115 °C (3 min); (B) incubated 65 °C (30 min) and heated 145 °C (4 min). Peak identification: 1, chlorophyllide *b*; 2, chlorophyllide *a*; 2′, chlorophyllide *a*′; 3, pheophorbide *b*; 3′, pheophorbide *b*′; 4, pheophorbide *a*; 4′, pheophorbide *a*′; 5, chlorophyll *b*; 5′, chlorophyll *b*′; 6, chlorophyll *a*; 6′, chlorophyll *a*′; 7, pheophytin *b*; 7′, pheophytin *b*′; 8, pheophytin *a*; 8′, pheophytin *a*′; 9, pyropheophytin *b*; 10, pyropheophytin *a*.

MS/MS analyses were carried out by B/E linked scanning, a type of measurement in which the magnetic field (B) is scanned in a constant ratio to the electrostatic analyzer voltage (E). Precursor ions, $M^{\cdot+}$, formed by FAB ionization, were fragmented by collisional activation using helium in the collision cell. The helium gas pressure was adjusted so that the abundance of the precursor ion was attenuated 70%. Mass spectra were recorded at constant B/E by the DA-5000 data system and plotted without background subtraction.

RESULTS AND DISCUSSION

The HPLC chromatograms shown in Figure 2, recorded at 658 nm, are representative of the chlorophyll derivatives which form during thermal treatments of spinach. Fresh tissue contains primarily chlorophylls *a* and *b*, while mild heat treatments induce epimerization at C-10 as well as loss of the centrally located Mg atom. Chlorophyllide formation, via hydrolysis of the C_{20} ester at C-7, can be catalyzed by activating the enzyme chlorophyllase (Lambers et al., 1984). High-temperature thermal treatments are necessary to induce loss of the C-10 β-keto ester group to produce the "pyro" derivatives. Although the C-10 epimers are resolved in the chromatograms shown in Figure 2 (labeled *a*′ and *b*′), only the parent compounds were isolated for mass spectrometric analysis. Details of the HPLC procedure to isolate these compounds with photodiode array spectral data have been published elsewhere (Canjura and Schwartz, 1991).

Molecular ion radicals, $M^{\cdot+}$, were observed in the positive ion FAB mass spectra of all 12 of the chlorophyll derivatives. For example, the positive ion FAB mass

Table I. Positive Ion FAB Mass Spectra of Chlorophyll Derivatives Using the FAB Matrix 3-Nitrobenzyl Alcohol

compd	$M^{\bullet+}$	B[a]	C	E	F	G
chlorophyll *a*	892.5		614.1	555.1		481.0
	(25)[b]		(100)	(39)		(29)
chlorophyll *b*	906.5		628.1	569.1		495.0
	(67)		(100)	(30)		(39)
pheophytin *a*	870.5		592.5	533.1	519.7	459.1[c]
	(100)		(53)	(41)	(36)	(83)
pheophytin *b*	884.5		606.1	547.0	533.0	473.0
	(100)		(37)	(31)	(15)	(23)
chlorophyllide *a*	614.3					
	(100)					
chlorophyllide *b*	628.2					
	(100)					
pheophorbide *a*	592.1	533.1		533.1		459.1[c]
	(100)	(17)		(17)		(16)
pheophorbide *b*	606.1	547.1		547.1		473.0
	(100)	(47)		(47)		(51)
pyropheophytin *a*	812.5		534.2		461.1[c]	
	(100)		(20)		(40)	
pyropheophytin *b*	826.3		548.1		475.1	
	(100)		(45)		(36)	
zinc pheophytin *a*	934.8		656.6	597.6		523.5
	(100)		(27)	(29)		(15)
zinc pyropheophytin *a*	876.8		598.8		525.6	
	(100)		(29)		(19)	

[a] See Figure 1 for explanation of fragmentation patterns A, B, C, etc. [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 set. [c] Contains contributions from the matrix cluster ion at m/z 460.

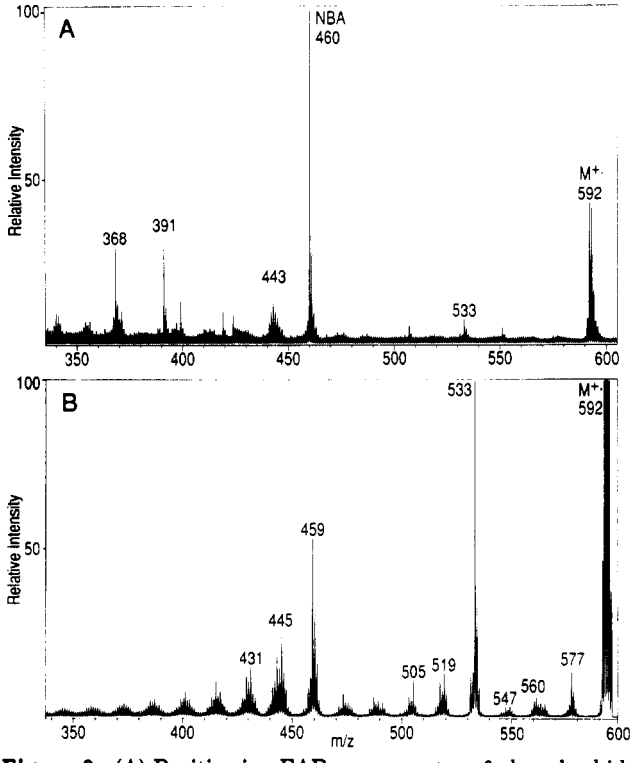

Figure 3. (A) Positive ion FAB mass spectra of pheophorbide *a* (**4a**) using a matrix of 3-nitrobenzyl alcohol (NBA). (B) *B*/*E* linked scan of the molecular ion of pheophorbide *a* (**4a**) using collisional activation. See Figure 1 for structure of **4a**.

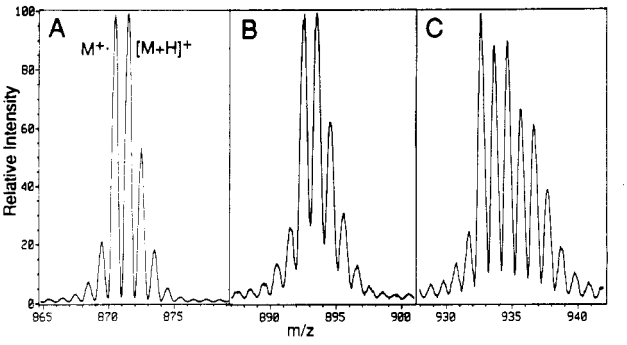

Figure 4. Molecular ion region of the positive ion FAB mass spectra of (A) pheophytin *a* (**2a**), (B) chlorophyll *a* (**1a**), and (C) zinc pheophytin *a* (**6**). See Figure 1 for structures of these chlorophyll derivatives.

spectrum of pheophorbide *a* is shown in Figure 3. The relative abundances of the molecular ions and most abundant fragment ions in the FAB mass spectra of each compound are summarized in Table I. Types of fragment ions common to the different chlorophyll derivatives are indicated in Figure 1. Except for chlorophylls *a* and *b*, for which loss of the phytyl chain (indicated as fragment ion type C in Figure 1) was the most abundant sample ion, molecular ions were more abundant than fragment ions. Loss of the phytyl chain (fragment ion C), sometimes with loss of additional methylene groups (fragment ions E and F) or with the additional loss of the β-keto ester group (fragment ion G), were the most abundant fragment ions. Because the pheophorbides lacked the phytyl chain, their most abundant fragment ions were type E, $[M - {}^{\bullet}CH_2CO_2H]^+$, or type B, which was loss of the β-keto ester group, $[M - {}^{\bullet}CO_2CH_3]^+$. The fragmentation of all chlorophyll derivatives was more extensive following collisional activation. These MS/MS results are discussed in more detail below.

Although matrix ions were present in all analyses, their relative abundances decreased as the amount of sample was increased. Typically, approximately 1 μg of chlorophyll derivative was loaded onto the FAB sample stage per analysis. Except when matrix ions were detected at the same m/z values as sample ions, these contaminants have been excluded from Table I.

Besides $M^{\bullet+}$ ions, chlorophyll derivatives also formed $[M + H]^+$ ions and, to a much lesser extent, $[M - H]^+$ ions during fast atom bombardment in the 3-nitrobenzyl alcohol matrix. Although $M^{\bullet+}$ ions were typically the most abundant molecular ion species, protonated molecules were almost as abundant as the molecular ion radicals in the FAB mass spectra of the pheophytins (Figure 4A), pheophorbides (Figure 3A), and pyropheophytins. Grese and co-workers (Grese et al., 1990) have compared the relative abundances of these three molecular ion species for chlorophyll *a* as a function of the FAB matrix and report

Table II. Positive Ion FAB Mass Spectra of Chlorophyll Derivatives Using *B/E* Linked Scanning with Collisional Activation

compd	$M^{\bullet+}$	$[M - {}^{\bullet}CH_3]^+$	A[a]	B	C	D	E	F	G	F − 14[c]	G − 14
chlorophyll *a*	892.5	877.6 (1)[b]	860.8 (1)	833.6 (2)	614.7 (100)	569.6 (2)	555.3 (17)	541.6 (5)	481.3 (22)	527.6 (3)	467.5 (8)
chlorophyll *b*	906.5	891.9 (1)	874.9 (1)	847.9 (1)	628.8 (100)	583.6 (2)	569.3 (18)	555.5 (3)	495.3 (16)	541.1 (3)	481.5 (3)
pheophytin *a*	870.8	855.6 (9)	838.4 (1)	811.7 (6)	592.8 (100)	547.6 (9)	533.1 (82)	519.7 (36)	459.1 (82)	505.6 (18)	445.5 (27)
pheophytin *b*	884.7	869.9 (5)	852.9 (8)	825.9 (5)	606.7 (100)	561.6 (8)	547.8 (92)	533.6 (31)	473.3 (46)	519.6 (15)	459.5 (15)
chlorophyllide *a*	614.3	599.1 (75)	582.1 (35)	555.1 (100)		569.4 (25)	555.1 (100)	541.1 (62)	481.2 (75)	527.1 (13)	467.1 (35)
chlorophyllide *b*	628.2	613.2 (24)	596.1 (29)	569.0 (100)		583.1 (10)	569.1 (100)	555.1 (21)	495.2 (86)	541.1 (17)	481.1 (20)
pheophorbide *a*	592.4	577.4 (15)	560.4 (6)	533.2 (100)		547.4 (3)	533.3 (100)	519.3 (13)	459.3 (56)	505.3 (11)	445.3 (26)
pheophorbide *b*	606.5	591.4 (24)	574.2 (24)	547.4 (100)		561.3 (23)	547.4 (100)	533.3 (29)	473.4 (53)	519.4 (41)	459.2 (41)
pyropheophytin *a*	812.5	797.7 (11)			534.9 (100)	489.4 (17)	475.4 (8)	461.4 (94)	401.4 (3)	447.4 (50)	387.5 (3)
pyropheophytin *b*	826.7	811.9 (12)			548.4 (88)	503.5 (24)	489.5 (12)	475.4 (100)	415.4 (12)	461.4 (56)	401.3 (24)
znic pheophytin *a*	934.8	919.8 (7)	902.8 (5)	875.8 (6)	656.6 (100)	611.7 (4)	597.6 (34)	583.6 (8)	523.5 (47)	569.5 (6)	509.5 (18)
zinc pyropheophytin *a*	876.8	861.8 (7)			598.8 (100)	553.6 (9)	539.6 (3)	525.6 (12)	465.5 (6)	511.6 (9)	451.5 (3)

[a] See Figure 1 for explanation of fragmentation patterns A, B, C, etc. [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 set. [c] F − 14 denotes a fragment ion of type F that has eliminated a methylene group weighing 14 mass units.

that the radical cation of chlorophyll *a* probably forms in solution during fast atom bombardment.

The chlorophyll derivatives containing magnesium, zinc, or neither metal could be distinguished from each other by molecular weight and by the isotope pattern of the molecular ions. If no metal was present, the molecular weight was lower and the isotope pattern was simpler than that of the derivatives containing magnesium or zinc. Because magnesium consists of three natural isotopes, ^{24}Mg (79.0%), ^{25}Mg (10.0%), and ^{26}Mg (11.0%), whereas there are five isotopes of zinc, ^{64}Zn (48.6%), ^{66}Zn (27.9%), ^{67}Zn (4.1%), ^{68}Zn (18.8%), and ^{70}Zn (0.6%), the molecular ion region of zinc pheophytin *a* and zinc pyropheophytin *a* was significantly broader than that of derivatives containing magnesium. For example, the molecular ion regions of pheophytin *a* (no metal), chlorophyll *a* (containing magnesium), and zinc pheophytin *a* are compared in Figure 4.

As shown in Figure 3A, matrix ions from the 3-nitrobenzyl alcohol and other chemical noise dominated the low-mass range of the mass spectra of pheophorbide *a* and the other chlorophyll derivatives. Following collisional activation of the molecular ion from each chlorophyll derivative, the fragment ions were recorded by using *B/E* linked scanning. Chemical noise and matrix ions were virtually eliminated from these MS/MS spectra as shown in the *B/E* linked scan of pheophorbide *a* in Figure 3B.

Although the *B/E* linked scans were obtained at unit resolution, several peaks were recorded for each fragment ion (Figure 3B). The heterogeneity of these ions was the result of limited resolution in selecting the precursor ion. Because a double-focusing mass spectrometer was used for these measurements instead of a three- or four-sector instrument, fragment ions were transmitted and recorded that were formed from a mixture of precursor ions. These ions consisted primarily of radical cations, $M^{\bullet+}$, but $[M + H]^+$ and $[M - H]^+$ ions as well as the isotope peaks of all three species contributed to the formation of fragment ions. Nevertheless, fragment ions formed from all 12 chlorophyll derivatives could be identified easily in the MS/MS spectra.

The m/z values and relative abundances of the major fragment ions obtained by collisional activation of $M^{\bullet+}$ precursors and *B/E* linked scanning for all 12 chlorophyll derivatives are listed in Table II. The fragmentation patterns, labeled A–G in Table II, are illustrated in Figure 1. These patterns are based on fragmentation pathways proposed by Chait and Field (1984) for chlorophyll *a* and confirmed by Grese et al. (1990).

Similar fragmentation patterns were observed for all of the different chlorophyll derivatives isolated from spinach tissue. For example, all species eliminated a methyl radical, $[M - {}^{\bullet}CH_3]^+$. Because the chlorophyllides and pheophorbides lacked the phytyl chain, loss of which was a major competing reaction in the other derivatives, elimination of a methyl radical was particularly favorable for these compounds. Other fragmentation pathways common to all chlorophyll derivatives were D, E, F, and F − 14. These involved fragmentation of the substituent group at C-7 prior to the site of attachment of the phytyl chain.

Fragment ions of the types A and B, $[M - CH_3OH]^{\bullet+}$ and $[M - {}^{\bullet}COOCH_3]^+$, respectively, were formed by fragmentation at the β-keto ester group at C-10 (Figure 1). These fragment ions were observed for all species except the pyropheophytins, which lacked a β-keto ester. The observation of fragment ions A and B is therefore indicative of the presence of the β-keto ester group at C-10. The chlorophyllides and pheophorbides formed the most abundant A and B fragment ions, with B ions accounting for the base peaks in the MS/MS spectra of these compounds. As in the case of the elimination of methyl radicals discussed above, the relative abundances of A and B ions in the mass spectra of the chlorophyllides and pheophorbides was enhanced by their lack of the phytyl chain, fragmentation of which would have been a major competing reaction. Other fragment ions indicative of the presence of the β-keto ester group were G and G − 14 ions. G ions were formed by elimination of the substituent groups at both C-7 and C-10 as indicated in Figure 1. Elimination of an additional 14 mass units (probably

a methylene group) from the G ion resulted in the less abundant G − 14 ions.

Fragmentation by pathway C was responsible for the formation of abundant fragment ions of all chlorophyll derivatives except the chlorophyllides and the pheophorbides. The C-type fragment ions, $[M-278]^{\bullet+}$, were formed by elimination of the phytyl chain with the transfer of a hydrogen from the leaving group back to the carboxylate oxygen to form a carboxylic acid. Because the chlorophyllides and pheophorbides have no phytyl chain and are already carboxylic acids, C ions were not observed in the collisionally activated MS/MS spectra of these compounds. Typically the most abundant fragment ion in the mass spectra of chlorophyll derivatives, the formation of $[M - 278]^+$ ions, is indicative of the presence of the phytyl chain.

Fragment ions D–F were formed by fragmentation similar to that producing the C ions but with additional loss of the carbonyl group. E and F ions also included elimination of methylene groups between the carbonyl carbon and C-7. Because the presence of the phytyl chain was not essential for the formation of these ions, the mass spectra of the chlorophyllides and pheophorbides also contained these fragment ions. The relative abundance of F and F − 14 ions for zinc pheophytin *a* and zinc pyropheophytin *a* was lower than the corresponding pheophytins and pyropheophytins and was similar to chlorophyll *a*, which contained Mg^{2+} instead of Zn^{2+}. Therefore, the presence of a divalent cation in chlorophyll derivatives containing the phytyl chain, whether Mg^{2+} or Zn^{2+}, reduced the formation of F ions.

CONCLUSIONS

The MS/MS spectra obtained by collisional activation of the molecular ions of a related series of 12 chlorophyll derivatives will provide a useful reference for the identification of unknown chlorophyll derivatives by mass spectrometry. Comparison of the fragmentation patterns for 12 compounds clearly shows the presence or absence of the phytyl chain and the β-keto ester. Other abundant fragment ions, D–F, further characterize the structure of the substituent group on C-7. In the analysis of an unknown chlorophyll derivative, the absence or modification of these groups could be determined by comparing the unknown mass spectrum to the data presented here and thus provide a sensitive technique for the analysis of these compounds from food and other biological tissues.

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