Improved Method To Track Chlorophyll Degradation

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An analytical method capable of identifying >30 chlorophyll-related compounds in plant extracts has been developed. The method employs liquid chromatography coupled to UV−vis, MS, and MS/MS detection. It can be applied without modification to analyze natural chlorophyll degradation products and other metalloporphyrines. It was successfully applied to identify chlorophyll derivatives found in rehydrated spinach powder and conventionally canned and Veri-Green-processed beans. In the Veri-Green-processed beans several degradation products were identified that are zinc-containing analogues to the chlorophyll derivatives found in vegetables after conventional canning. They have been characterized by liquid chromatography and mass spectrometry.

Keywords: *Chlorophyll; food; beans; spinach; degradation; vegetables; analytical; LC; MS; MS/MS; fragmentation; spectra; processing*

INTRODUCTION

The degradation of chlorophyll during the processing of fruits and vegetables is still an industrial challenge. When the natural cellular structures are disintegrated upon industrial processing, the pigment becomes amenable to various enzymatic and nonenzymatic reactions, which finally lead to brownish degradation products. As consumers perceive the green color of chlorophyll as an indicator of the "freshness" of food, many studies have been carried out to understand and to control the degradation of this molecule.

In green tissues subjected to various conditions of processing, chlorophyll undergoes distinct types of degradation reactions. Demetalation and epimerization are observed during heat treatment (*1*, *2*), and prolonged heating leads to additional demethoxycarbonylation of the molecule (*3*). Demethoxycarbonylation also occurs during the canning of vegetables (*4*). The dephytylation of chlorophyll is achieved enzymatically during, for example, fermentation (*5*) and storage (*6*). Dephytylation is often observed together with demetalation (*5*, *6*). Allomerization is observed during the preparation of table olives (*7*) and was also reported during boiling (*8*).

Efforts to improve the quality of processed green vegetables through the retention of chlorophyll have been extensive, although unsuccessful. Formation of green metallocomplexes of chlorophyll derivatives during thermal processing, known as "regreening", is considered to be a promising method to preserve the color of canned green vegetables (*9*). As an example, the Veri-Green process is a patented procedure by which blanching of green vegetables is performed in the presence of zinc(II) salts (*10*). The regreening of Veri-Green-processed beans has mainly been attributed to the formation of zinc complexes of pheophytin and pyropheophytin (*11*), although the chromatographic conditions used did not allow the separation of pheophytin *b* and Zn-pyropheophytin *a*.

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In the past, investigations of these rather complex reactions were hampered by a lack of suitable analytical methods. The enhancement of analytical tools, such as the coupling of high-performance liquid chromatography (HPLC) to mass spectrometry (MS), opened new perspectives in chlorophyll research. First experiments on the analysis of chlorophyll and their derivatives by HPLC-MS were carried out using continuous flow fast atom bombardment (*12*−*15*) and thermospray ionization (*16*). The use of atmospheric pressure ionization (API) techniques improved the assignment of chlorophyll derivatives further (*17*). However, despite these individual approaches, up to now no complete analytical method has been described that enables, in a short time, the separation and simultaneous identification of most chlorophyll species in processed plant tissues.

The scope of the present paper is to outline the development of an analytical method for the identification of a wide range of chlorophyll species. Its application is demonstrated by analyzing pigment extracts of rehydrated spinach powder as well as comparing chlorophyll derivates found in conventionally canned beans and Veri-Green-processed beans.

EXPERIMENTAL PROCEDURES

Chemicals. All solvents were of gradient grade and were purchased from Merck (Geneva, Switzerland). Chlorophylls *a* and *b* were from Extrasynthèse (Genay, France).

Preparation of Allomerization Products. The method was adapted from that of Pennington et al. (*18*). One hundred micrograms of chlorophyll *a* was dissolved in 3.5 mL of methanol that was dried over a 0.3 nm molecular sieve saturated with pure oxygen. The solution was stirred at room temperature in the dark. Aliquots were taken at defined intervals, and the chlorophyll content was analyzed by HPLC (see below). When the reaction was complete, the methanol was evaporated to dryness under argon. The products were redissolved in cyclohexane and stored at −18 °C.

Material. Spinach leaves were purchased from local growers. For powder preparation, the spinach was freeze-dried and the dried leaves were ground with a pestle and mortar. Powders were rehydrated in water (powder/water = 1:9, w/w) for 1 day. Conventionally canned beans and Veri-Green-processed beans were a gift from Crown Cork and Seal.

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Table 1. Peak Assignment of Chlorophylls Separated by RP-HPLC and Shown in Figures 1, 3, and 4

peak	assignment	abbreviation	retention time (min)	expected color	quantification coefficient ($\times 10^{-3}$)	identification	$[M + H]^+$	main fragment ions
1	chlorophyllide *b*	CHLIDb	6.7	green	0.208	standard		
2	unknown b	Ukn b12	12.0	?				
3	chlorophyllide *a*	CHLIDa	13.6	green	0.850	standard		
4	chlorophyllide *a*′	CHLIDa′	14.3	green	0.850	UV[a]		
5	unknown a	Ukn a14	14.6	?				
6	pyrochlorophyllide *a*	PyrCHLIDa	17.0	green	0.850	UV[b]		
7	unknown a	Ukn a18	17.8	brown				
8	pheophorbide *b*	PHEIDb	18.1	brown	2.507	standard; MS		
9	pheophorbide *a*	PHEIDa	20.4	brown	1.353	standard; MS	593.2 (100)	575.3 (35), 535.2 (15), 517.2 (70)
10	pheophorbide *a*′	PHEIDa′	21.1	brown	1.353	UV[a]		
11	pyropheophorbide *a*	PyrPHEIDa	22.8	brown	1.353	UV,[a] MS[c]	549.2 (100)	527.2 (50), 501.1 (65)
12	hydroxychlorophyll *b*	CHLb1/OH-CHLb	25.5	green	0.699	MS[c]		
13	unknown b	Ukn b25	25.9	?				
14	chlorophyll *b*	CHLb	26.6	green	0.699	Purum,[d] MS	907.4 (100)	629.1 (35)
15	chlorophyll *b*′	CHLb′	27.1	green	0.699	UV[a], MS	907.4 (100)	629.1 (35)
16	hydroxychlorophyll *a*	CHLa1/OH-CHLa	27.6	green	0.305	MS[c]	909.6 (20)	891.5 (100), 631.2 (10), 613.2 (15)
17	chlorophyll *a*	CHLa	29.3	green	0.305	Purum,[d] MS	893.5 (100)	615.1 (15)
18	chlorophyll *a*′	CHLa′	30.1	green	0.305	UV,[a] MS	893.5 (100)	615.1 (15)
19	unknown a	Ukn a33	33.8	?				
20	hydroxypheophytin *a*	PHEa1	38.1	brown	0.497	UV,[a] MS[c]	887.4 (100)	869.5 (55), 609.2 (10), 591.1 (15)
21	pheophytin *b*	PHEb	39.6	brown	1.209	standard, MS	885.4 (100)	607.2 (25)
22	pheophytin *a*	PHEa	45.6	brown	0.497	standard, MS	871.5 (100)	593.2 (10)
23	pheophytin *a*′	PHEa′	48.9	brown	0.497	UV,[a] MS	871.5 (100)	593.2 (10)
24	unknown a	Ukn a11	11.7	?				
25	pheophorbide *b*′	PHEIDb′	18.7	brown	2.507	UV[a]		
26	pyropheophorbide *b*	pyrPHEIDb	20.4	brown	2.507	MS[c]	535.2 (100)	513.2 (15), 487.1 (10)
27	methoxychlorophyll *a*	CHLa2/MeO-CHLa	28.2	green		MS[c]	923.1 (15)	891.3 (100)
28	methoxylactone chlorophyll *a*	MeO-L-CHLa	28.7	green		MS[c]	939.5 (100)	924.5 (5), 907.5 (7), 891.4 (25), 660.9 (10)
29	hydroxypheophytin *a*′	OH-PHEa′	42.1	brown	1.209	UV,[a] MS		
30	pheophytin *b*′	PHEb′	41.5	brown	1.209	UV,[a] MS		
31	pyropheophytin *b*	pyrPHEb	55.1					
32	pyropheophytin *a*	pyrPHEa	68.2	brown	1.209	UV,[a] MS	813.8 (100)	535.6 (5)
33	unknown	Ukn a17	17.3	?		MS	572 (100)	516 (100), 498 (20), 353 (35), 335 (25)
34	unknown	Ukn a19	19.4	?		MS	572 (100)	353 (90), 261 (50), 243 (35)
35	unknown	Ukn a20	20.6	?		MS	712 (70)	335 (100)
36	Zn-pheophytin *a*	Zn-PHEa	33.1	green		MS	933.5 (100)	655 (100), 595 (50)
37	Zn-pheophytin *a*′	Zn-PHEa′	35.1	green		MS	933.5 (100)	655 (100), 595 (50)
38	Zn-pyropheophytin *a*	Zn-pyrPHEa	36.4	green		MS	875.5 (100)	597 (100)

[a] Identification by comparison of UV spectrum with the corresponding "parent" standard. [b] Tentative identification of demethoxycarbonylated derivatives of dephytylated species by UV; see text. [c] Identification by LC-MS and additional comparison with retention time of standard. [d] Solution of pure standard (Fluka, >95%).

Pigment Extraction from Plant Material. Rehydrated spinach powder and dripped-off beans were ground in 4 volumes of cold acetone. Insoluble proteins were precipitated by spinning (Biofuge A, Heraeus Christ, Carouge, Switzerland) after the extract had been stored at −18 °C for 30 min. Sonication was used to dissolve any recalcitrant chlorophyll. Aliquots of the supernatant were immediately used for HPLC injection or stored at −40 °C for a maximum of 48 h.

HPLC-Vis. Separation by reversed-phase HPLC was performed on a Lichrospher 100-RP-18 C18-5 μm, 250 × 4.6 mm, column (Bischoff, Leonberg, Germany). A Shimadzu (Kyoto, Japan) HPLC system, consisting of an SIL-10A autosampler, an LC-10AD solvent delivery module, and a nSPD-M10AV UV−vis photodiode array detector, was used. UV absorption was recorded at 430, 650, and 670 nm. Solvent A was prepared from 1 M aqueous ammonium acetate and methanol (1:4, v/v), and solvent B was acetone/methanol (1:4, v/v). The separation was adapted from that of Zapata et al. (*19*). At a flow rate of 1.0 mL/min, a linear gradient was run at room temperature from 100% A to 100% B in 15 min and held at 100% B for a further 30 min. The column was then returned to initial conditions over 2 min and equilibrated with 100% A for 8 min. When the presence of pyrPHE was expected in the samples, the isocratic part of the run (at 100% B) was maintained for 50 min instead of 30 min.

HPLC-MS and HPLC-MS/MS. Separation was performed using a Waters HPLC system, consisting of a type 757 autosampler, a 600-MS pump with system controller, and a type 486-MS UV detector. UV absorption at 430 nm was recorded using an analogue input to the mass spectrometer's data system. The same column as above was used; however, the gradient was changed slightly to achieve compatibility with the MS operating conditions. Solvent A was 0.1 M ammonium acetate, solvent B was methanol, and solvent C was acetone. Using a flow rate of 1 mL/min the separation started with a linear gradient from 20% A and 80% B, reaching 80% B and 20% C in 15 min and continued with an isocratic run for 30 min. The composition was changed to 45% B and 55% C after 50 min and kept constant for a further 10 min. Initial conditions were reached within 5 min and were maintained for 5 min to re-equilibrate the column.

The mass spectrometer was a Finnigan TSQ 700 triple-quadrupole mass spectrometer (San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) source. Data acquisition was performed on a DECstation 2100 running under Ultrix 4.2A (Digital Equipment) using the Finnigan software package ICIS2, version 7.0. The vaporizer temperature was set to 450 °C and the transfer capillary to 150 °C. The corona discharge current was set to 5 μA. Mass spectra were acquired in positive mode by scanning from *m*/*z* 200 to 1500 in 2 s. Daughter ion spectra were obtained at various collision energies (−10 to −30 eV in the laboratory frame) using argon at a pressure of 0.4 Pa (3 mTorr) as the collision gas.

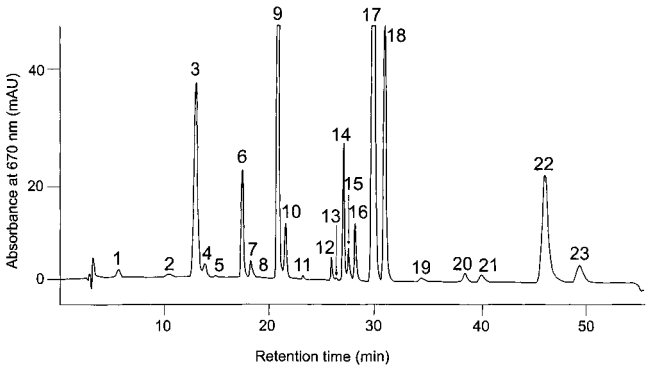

Figure 1. Typical chromatogram of chlorophylls extracted from rehydrated spinach powder, separated by RP-HPLC and detected by DAD at 670 nm. Peak numbers refer to Table 1. The separation conditions are described in the text.

RESULTS AND DISCUSSION

Characterization of the Chlorophyll Derivatives. The procedure described above enabled the separation (Figure 1) and identification of >25 chlorophyll-related compounds (Table 1) within a single run. The identities of the individual components were verified by HPLC-MS and were then, for routine use, assigned by comparison of their retention time and their UV spectra with that of known standards.

The mass spectra of the identified chlorophyll species are characterized by intense protonated molecular ions. Upon MS/MS (Figure 2), a major fragment at (M + H − 278) is observed with all chlorophyll derivatives that possess the phytyl chain. Depending on the ionization conditions, this fragment can already be observed in HPLC-MS, albeit at lower abundance. It corresponds to the loss of the phytyl moiety as phytadiene, $C_{20}H_{38}$, and is formed by elimination of the phytyl chain with hydrogen transfer from the leaving group back to the carboxylate oxygen to form the carboxylic acid (*12*). Another characteristic fragment is obtained at (M + H − 338), probably corresponding to elimination of $CH_3COOC_{20}H_{39}$.

As the *a*/*a*′ and the *b*/*b*′ species are configuration isomers, their MS/MS fragmentation patterns are basically identical. However, we observed that the abundance of fragment ions is different for the two families: under identical conditions, the *a* and *b* species show approximately equal intensities for the two main fragment ions, with the $[M + H]^+$ still being the base peak (Figure 1a,c). In contrast to this, the *a*′ and *b*′ isomers exhibit an abundant $[M + H - 78]^+$ ion, whereas the $[M + H - 338]^+$ and $[M + H]^+$ ions occur with minor intensity (Figure 2b,d). In addition, a characteristic fragmentation common to the *b* derivatives is the formation of an $[M + H - 28]^+$ ion, which corresponds most likely to a loss of CO from the aldehyde function on C-3 (Figure 2b,d). Similarly, the spectrum of pyropheophytin *b* shows $[M + H]^+$ and $[M + H - 278]^+$ ions accompanied by $[M - 28]^+$ ions. Furthermore, it was found that the hydroxylated compounds are characterized by an intense ion at $[M - 17]^+$, corresponding to loss of water from the protonated molecular ion.

Figure 1 shows the separation of chlorophyll derivatives from an acetone extract of rehydrated spinach powder. The UV spectra of each peak showed the typical UV−vis spectrum of chlorophyll (data not shown). Detection at 670 nm for chlorophyll species of the *a* type and at 650 nm for chlorophyll species of the *b* type avoids interference from xanthophylls and carotenoids. Chlorophylls *a* and *b* were identified by LC-MS as peaks 17 and 14, respectively, and pheophytins *a* and *b* as

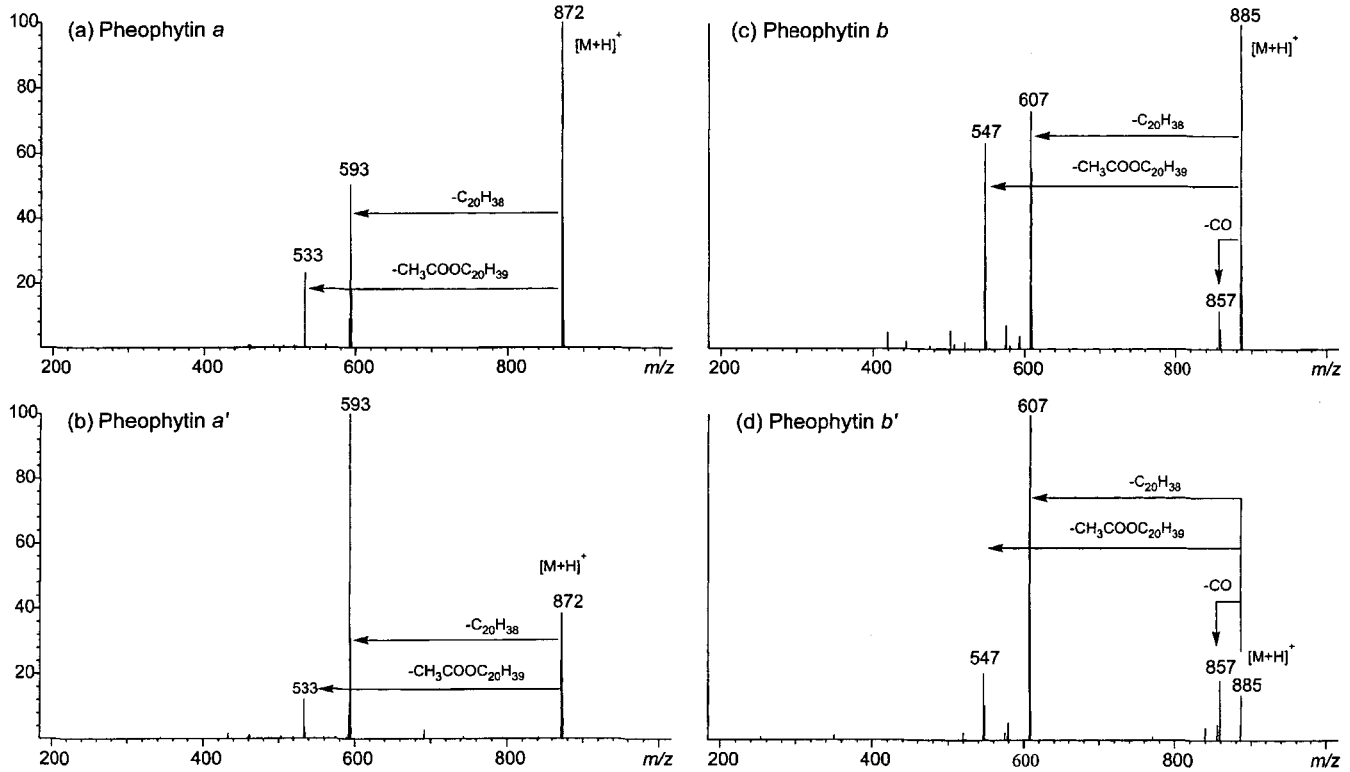

Figure 2. Daughter mass spectra of the $[M + H]^+$ ions of four pheophytins. The structural difference between the *a*/*b* and the a′/*b*′ isomers is reflected in the fragment ion intensities (panels a and c versus panels b and d). Note the loss of CO, which is characteristic for the *b* chlorophylls (c, d).

Table 2. Identification of the Allomerization Products of Chlorophyll *a* in Methanol

peak	assignment	abbreviation	retention time (min)	absorbance max	$[M + H]^{+}$ [a]	main fragment ions[a]
1	unknown		26.96	421/657		
2	hydroxychlorophyll *a*	OH-CHLa	27.39	431/664		
3	methoxychlorophyll *a*	MeO-CHLa	27.70	423/662	923.1 (15)	891.3 (100)
4	methoxylactone chlorophyll *a*	MeO-L-CHLa	28.02	421/657	939.5 (100)	924.5 (5), 907.5 (7), 891.4 (25), 660.9 (10)
5	chlorophyll *a*	CHLa	28.99	431/664	893.5 (100)	615.1 (15)
6	chlorophyll *a*′	CHLa′	30.08	431/664	893.5 (100)	615.1 (15)

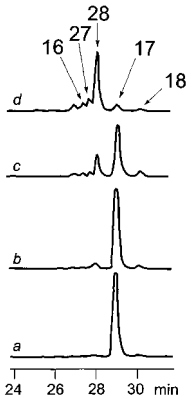

Figure 3. HPLC separation of the allomerization products of chlorophyll *a* formed in methanol (see text) after 0 min (a), 2 h (b), 8 h (c), and 24 h (d). See Table 1 for abbreviations.

peaks 22 and 21, respectively. These identifications were confirmed with pure standards.

HPLC-MS enabled the characterization of pheophorbides *a* and *b*, indicated as peaks 9 and 8 in Figure 1, respectively. However, due to the need for 0.1 M ammonium acetate buffer in HPLC-MS, chlorophyllides were not sufficiently retained and eluted in the solvent front. The high polarity of the chlorophyllides impeded good interaction with the stationary phase, necessitating high concentrations of ion-pairing salts (*20*, *21*), such as 1 M ammonium acetate. Thus, chlorophyllides *a* and *b* were attributed to peaks 3 and 1, respectively, according to their retention times and UV spectra.

The separation and detection of chlorophyll allomers have been another problem encountered in earlier investigations (*22*). Allomerization of chlorophyll *a* in methanol was used to produce chlorophyll allomers. After 24 h, chlorophyll *a* was almost entirely transformed into methoxylactone chlorophyll *a* as shown in Figure 3, and five compounds were identified as listed in Table 2. The polarities of these allomers are close to each other, and most of them are essentially indistinguishable from their parents in the UV−vis spectra (*23*−*26*) due to the peripheral positioning of allomerization sites (*22*). Kuronen et al. (*27*) mentioned that the complete allomerization takes ~3 days. We saw that a much faster reaction could be observed in our studies after stirring in water-free, oxygenated methanol. As observed earlier (*28*), the presence of water inhibits the reaction, probably through the stabilization of the enol form by hydrogen bonding.

Application To Identify Chlorophyll Derivatives Found in Conventionally Canned and Veri-Green-Processed Beans. Figure 4 shows the chromatograms obtained with pigment extracts of conventionally canned beans (a) and Veri-Green-processed beans (a). In comparison to the pigments found in rehydrated spinach powder (Figure 1), no substantial amounts of chlorophyll *a* or *b* could be detected in the acetone extracts. In the conventionally canned beans we found pheophytin *a* and pyropheophytin as the most prominent products of chlorophyll degradation degradation, which is in agreement with previous findings (*3*, *4*). In contrast to this, the Veri-Green-processed beans show a group of three distinct peaks related to zinc-containing degradation products. They elute between 30 and 40 min and are indicated as peaks 36−38 in Figure 4b.

Peak 36 was identified as Zn-pheophytine *a*. The ion observed at *m*/*z* 933.5 (Figure 5a) corresponds to the $[M + H]^{+}$ of chlorophyll *a*, where Mg was exchanged against Zn and which is further confirmed by the characteristic isotope pattern. Its identity was confirmed further by an MS/MS daughter ion scan of *m*/*z* 933.5 (Figure 5b), which shows the two characteristic fragment ions described above for chlorophyll *a*: (M + H − 278) due to loss of $C_{20}H_{38}$ from the phytyl group and (M + H − 338), probably elimination of $CH_3COOC_{20}H_{39}$. To a small extent, *m*/*z* 655 is already formed in the ion source (Figure 5a).

Peak 37 shows the same ions as peak 36, but with a lower abundance. As the chromatographic behavior resembles that of the chlorophyll *a* and *a*′ species, we can safely assume that this signal corresponds to Zn-pheophytin *a*′.

Peak 38 was identified as Zn-pyropheophytin *a*, supported by the observed ion *m*/*z* 875.5 and the characteristic isotope pattern of Zn and the chromatographic behavior (elution shortly after the pheophytins).

It should be noted that a peak group similar to 36−38 is observed earlier in the chromatogram (peaks 33−35 in Figure 4b). Again, the first two peaks show ions with identical *m*/*z* (572) and very similar MS/MS data, and the third peak is a compound with lower mass (*m*/*z* 478 and 518). However, we were not able to attribute their identity unambiguously.

In addition to the findings of von Elbe et al. (*11*), the chromatographic conditions described here allow the separation of additional zinc complexes of chlorophyll from Veri-Green-processed beans. Within the group of phytylated metallochlorophylls, we could clearly identify Zn-pheophytin *a*, Zn-pheophytin *a*′, and Zn-pyropheophytin as the main zinc-containing chlorophyll derivatives. In addition, our results suggest the presence of dephytylated zinc complexes of chlorophyll at low abundance in the "regreened" beans. Indeed, it has already been shown that dephytylated chlorophyll derivatives could react even more quickly with zinc(II) ions than phytylated chlorophylls (*29*). However, for the formation of such polar chlororophyll compounds in "regreened" beans, it is not clear if the phytol has been lost before or after complexation with the zinc ion.

Conclusions. We have developed an analytical method that allows the identification of >25 chlorophyll-related compounds in vegetable extracts. Using HPLC

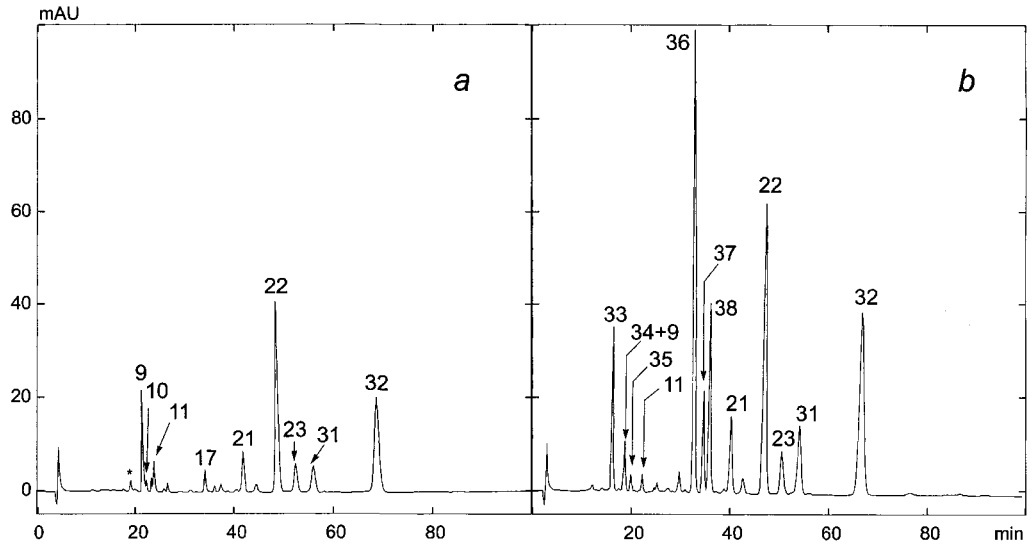

Figure 4. HPLC-UV trace from (a) canned beans and (b) Veri-Green-treated canned beans. See text for discussion and Table 1 for peak identification.

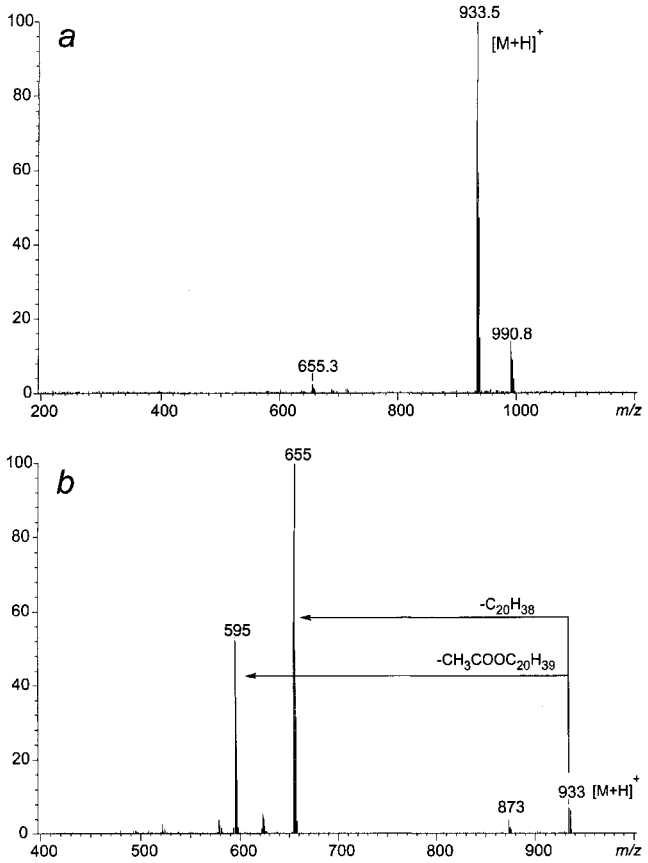

Figure 5. (a) Mass spectrum of peak 36 from Figure 4; (b) MS/MS daugther ion spectrum, obtained from the ion *m*/*z* 933.5 in (a). See text for discussion.

coupled to UV−vis, APCI-MS, and MS/MS detection, chlorophyll degradation products can be tracked quantitatively. The method was used successfully to identify chlorophyll derivatives in rehydrated spinach powder and canned and Veri-Green-processed beans. The procedure described herein not only allows the investigation of natural chlorophyll degradation products but can also be applied without modification to other metalloporphyrins. As an example, the study of Veri-Green-processed beans revealed the presence of several zinc pheophytins having chromatographic and mass spectrometric behaviors parallel to those of their magnesium-containing counterparts. It should be noted that some of these peaks have not been separated or reported in previous work (*11*). These results indicate that our method is a powerful tool to investigate the effect of several types of processing on chlorophyll degradation in green vegetables.

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