

Identification and Quantification of Metallo–Chlorophyll Complexes in Bright Green Table Olives by High-Performance Liquid Chromatography–Mass Spectrometry Quadrupole/Time-of-Flight

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ABSTRACT: Five different samples of table olives, two regular Spanish table olives and three “bright green table olives”, have been analyzed by HPLC–MS/MS to determine their pigment profile. Typical pigment profiles of almost all table olives show primarily chlorophyll derivatives lacking metals (e.g., pheophytin *a/b* and 15²-Me-phytol-chlorin *e*₆). Bright green table olives have a unique profile including metallo–chlorophyll complexes (Cu-15²-Me-phytol-chlorin *e*₆ with 26–48% and Cu-pheophytin *a* with 3–18%) as their major pigments. New tentative structures have been identified by MS such as 15²-Me-phytol-rhodin *g*₇, 15²-Me-phytol-chlorin *e*₆, 15²-Me-phytol-isochlorin *e*₄, Cu-15²-Me-phytol-rhodin *g*₇, Cu-15²-Me-phytol-chlorin *e*₆, and Cu-15²-Me-phytol-isochlorin *e*₄, and new MS/MS fragmentation patterns are reported for Cu-15²-Me-phytol-rhodin *g*₇, Cu-15²-Me-phytol-chlorin *e*₆, Cu-pheophytin *b*, Cu-pheophytin *a*, Cu-pyropheophytin *b*, and Cu-pyropheophytin *a*. The presence of metallo–chlorophyll derivatives is responsible for the intense color of bright green table olives, but these metallo–chlorophyll complexes may be regarded as a “green staining” defect that is unacceptable to consumers.

KEYWORDS: high-performance liquid chromatography, mass spectrometry, table olives, Cu-pheophytin, metallo–chlorophylls, green staining alteration, regreening

INTRODUCTION

The color of vegetable products is one of the most important sensory attributes evaluated by consumers worldwide, and, in green fresh vegetables and fruits, the green color is due to the presence of chlorophylls *a* and *b*. For table olives, color is a very critical attribute.

The most common color changes of fresh vegetables during cooking, freeze preservation, storage, or heat processing are due to the conversion of chlorophylls (*a* and *b*), responsible for bright green color, into their respective pheophytins (*a* and *b*) that are responsible for yellow-brown olive color. This dramatic change in color involves the release of the magnesium from the porphyrin ring. However, the insertion of metals within the porphyrin ring, such as zinc (Zn²⁺) or copper (Cu²⁺), produces a regreening effect. These metallo–chlorophyll complexes are more stable, due to higher metal–porphyrin bond energies, and more resistant to acid and heat than are naturally occurring magnesium (Mg²⁺) chlorophyll complexes.¹ In addition, the formation of copper complexes is much more rapid than zinc complexes.^{2,3} Regreening via the formation of metallo–chlorophyll complexes has been used to preserve the desirable fresh green color of vegetables by the food industry,^{4,5} including several patents.^{4–7}

In the traditional processing of Spanish style of table olives, chlorophylls *a* and *b* are degraded completely to pheophytins and pheophorbides during the fermentation process by two different and coexisting mechanisms. In the initial treatment of the olives with NaOH, at alkaline pH, the chlorophyllase is active, but in the fermentation process, pH turns acidic followed by the loss of Mg²⁺. The pigment transformation brought about by

Table 1. Gradient Used for Elution of Pigments by HPLC Reverse Phase^a

time (min)	solvent A	solvent B	solvent C	flow (mL/min)	curve
0.00	75.0	25.0	0.0	1.25	1
8.00	25.0	75.0	0.0	1.25	6
10.00	25.0	0.0	75.0	1.25	6
11.00	19.0	0.0	81.0	1.25	6
13.00	14.0	0.0	86.0	1.25	6
15.00	11.0	0.0	89.0	1.25	6
16.00	0.0	0.0	100.0	1.25	6
18.00	0.0	0.0	100.0	1.25	6
20.00	75.0	25.0	0.0	1.25	1

^a (1/1/8) (v/v/v) water/ammonium acetate 1 M/methanol (solvent A), acetonitrile (solvent B), and reagent alcohol (solvent C).

lactic fermentation produces olives that are highly valuable for their characteristic golden color.⁸

The innovations introduced in the traditional system of processing (elimination of the short washing, addition of acids, reuse of brines, etc.) have changed the previously described mechanism of chlorophyll degradation, with the detection of oxidative reactions that affects the chlorophyll isocyclic ring and production of allomerized chlorophylls.⁹

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Nevertheless, the Gordal variety, used almost exclusively to produce table olives, develops an alteration in color, known as “green staining” (GS) that is undesirable to consumers.¹⁰ This common defect is observed as bluish-green zones distributed over the skin that contrasts with its natural olive color. This alteration most likely occurs through the formation of endogenous metal porphyrin complexes to create GS.¹¹ The formation of metallo–chlorophyll complexes is thought to arise from the fruit’s endogenous Cu and may involve degradation of cell integrity allowing contact of the metal with the chlorophyll derivatives, which under normal conditions are protected in their natural lipophilic environment. Endogenous metal such as Cu may arise from the pectin chain that can serve as a reservoir of copper that is captured by chlorophyll pigments separated

from the thylakoid membrane to form the copper chlorophyll complexes.¹²

The most common technique to analyze these pigments is RP-HPLC-DAD, although HPLC–MS has also been employed for the determination of chlorophylls and their derivatives because it provides structural information, and, in addition, it is a very sensitive and selective technique. Methods based on HPLC–MS include atmospheric pressure ionization (APCI)^{13,14} and electrospray ionization (ESI)^{15,16} interfaces.

Previously, GS alteration has been studied,^{8,11} although those studies did not utilize HPLC–MS and tandem MS with accurate mass as an identification tool. In this study, metallo–chlorophyll complexes responsible for green staining are identified by HPLC mass spectrometry and quantified by HPLC in three commercial samples. In addition, their pigment profiles are compared to typical table olives (yellow-brown olive color).

MATERIALS AND METHODS

Samples. The study was carried out with five different commercial green table olives bought at different markets: sample A, “Italian Castelvetrano Whole Green Olives” (product of Italy) packed by Mezzetta Inc.; sample B, “Olives Castelvetrano Divina” packed by Whole Foods Market; sample C, “Green Table Olives” packed by Vincenzo’s; sample D, “Spanish Olives” (*var.* Manzanilla); and sample E, “Spanish Queen Olives” (*var.* Gordal) (both Spanish products) distributed by Kroger Co.

Chemicals and Standards. Ammonium acetate was supplied by Fluka (Zwijndrecht, The Netherlands). HPLC/MS and HPLC reagent grade solvents were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Standards of pheophorbide *a*, Cu-pheophorbide *a*, rhodin *g*₇, chlorin *e*₆, and Cu-chlorin *e*₆ were supplied by Porphyrin Product Inc. (Logan, UT). Chlorophylls *a* (chl *a*) and *b* (chl *b*) were

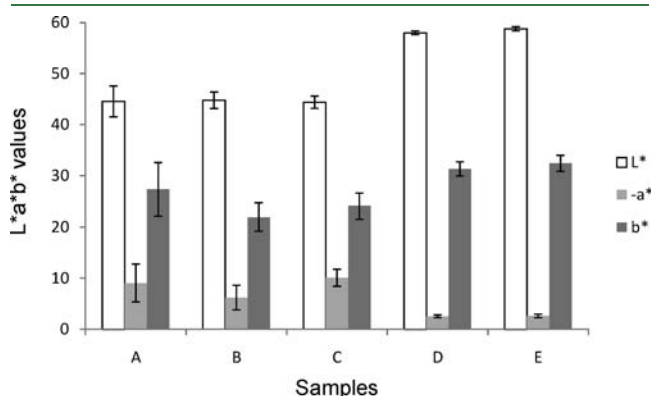


Figure 1. Color measurement $L^*a^*b^*$ coordinates of five samples of table olives.

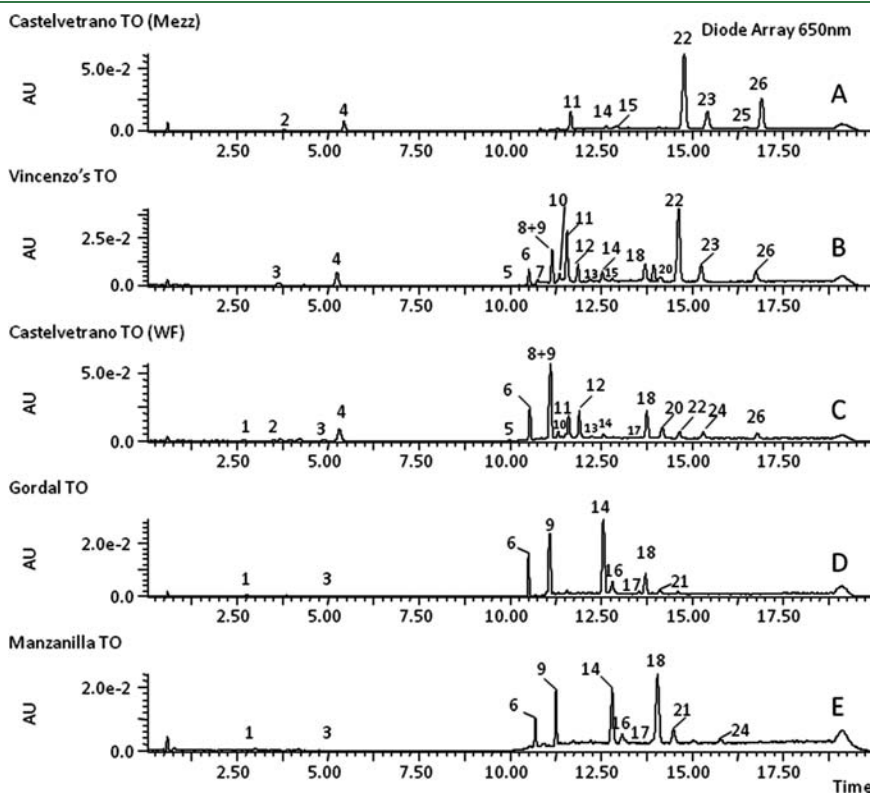
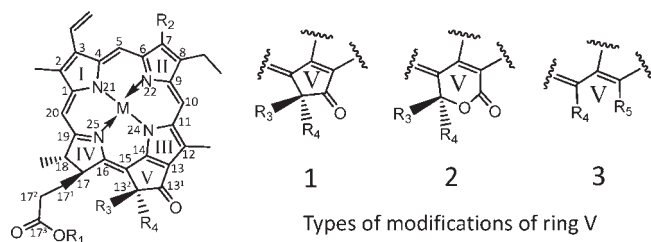


Figure 2. HPLC chromatograms of five samples, coded A–E, recorded at 650 nm. Note: AU, absorption units; TO, table olives.

Table 2. Different Structures of Pigments



pigment	I. ring ^a	R ₁	R ₂	R ₃	R ₄	R ₅	M
pheophorbide <i>a</i>	1	H	CH ₃	H	COOCH ₃		2H
pyropheophorbide <i>a</i>	1	H	CH ₃	H	H		2H
Cu-pheophorbide <i>a</i>	1	H	CH ₃	H	COOCH ₃		Cu
Cu-pyropheophorbide <i>a</i>	1	H	CH ₃	H	H		Cu
15 ² -Me-phytyl-rhodin <i>g₇</i> ester	3	phytyl	CHO		CH ₂ COOCH ₃	COOH	2H
Cu-15 ² -Me-phytyl-rhodin <i>g₇</i> ester	3	phytyl	CHO		CH ₂ COOCH ₃	COOH	Cu
chlorophyll <i>b</i>	1	phytyl	CHO	H	COOCH ₃		Mg
15 ² -Me-phytyl-chlorin <i>e₆</i> ester	3	phytyl	CH ₃		CH ₂ COOCH ₃	COOH	2H
Cu-15 ² -Me-phytyl-chlorin <i>e₆</i> ester	3	phytyl	CH ₃		CH ₂ COOCH ₃	COOH	Cu
chlorophyll <i>a</i>	1	phytyl	CH ₃	H	COOCH ₃		Mg
pheophytin <i>b</i>	1	phytyl	CHO	H	COOCH ₃		2H
Cu-pheophytin <i>b</i>	1	phytyl	CHO	H	COOCH ₃		Cu
pyropheophytin <i>b</i>	1	phytyl	CHO	H	H		2H
Cu-pyropheophytin <i>b</i>	1	phytyl	CHO	H	H		Cu
pheophytin <i>a</i>	1	phytyl	CH ₃	H	COOCH ₃		2H
Cu-pheophytin <i>a</i>	1	phytyl	CH ₃	H	COOCH ₃		Cu
pyropheophytin <i>a</i>	1	phytyl	CH ₃	H	H		2H
15 ² -Me-phytyl-isochlorin <i>e₄</i>	3	phytyl	CH ₃		CH ₂ COOCH ₃	H	2H
15 ² -Et-phytyl-chlorin <i>e₄</i>	3	phytyl	CH ₃		CH ₂ CH ₃	COOH	2H
Cu-15 ² -Me-phytyl-isochlorin <i>e₄</i>	3	phytyl	CH ₃		CH ₂ COOCH ₃	H	Cu
Cu-15 ² -Et-phytyl-chlorin <i>e₄</i>	3	phytyl	CH ₃		CH ₂ CH ₃	COOH	Cu
Cu-pyropheophytin <i>a</i>	1	phytyl	CH ₃	H	H		Cu

^a I. ring: Isocyclic ring (ring V).

supplied by Sigma-Aldrich Co. (St. Louis, MO). Standards of pheophytin *a* (phy *a*) and *b* (phy *b*) were obtained from their respective chlorophyll by acidification.¹⁷ Standards of Cu-pheophytin *a* and *b* were obtained from their respective chlorophyll by reaction with an excess of copper(II) ions as chloride.¹⁰

Extraction of Pigments. Fifty depitted fruits (ca. 40 g) were ground and homogenized. Triplicate extractions were prepared by accurately weighing 5–15 g for each sample. Pigment extraction was performed with *N,N*-dimethylformamide (DMF), according to the method proposed by Mínguez-Mosquera and Garrido-Fernández¹⁸ that allows the selective separation of pigments in oil free pigment solutions. Thus, DMF retains chlorophyll and xanthophylls, while the hexane phase retains the carotene fraction. All of the analytical procedures were performed under red lighting to avoid any photooxidation of the chlorophyllic compounds.

HPLC–MS and HPLC–MS/MS. HPLC–MS analysis was carried out on a Waters 2695 gradient HPLC separation module (Waters Corp., Milford, MA) equipped with an auto injector and a 996-photodiode array detector (PDA). Chromatographic separations were performed on a HALO C18 Symmetry column (75 mm × 4.6 mm i.d., 2.7 μm) (MAC-MOD Analytical, Inc.). The mobile phase consisted of (1/1/8) (v/v/v) water/ammonium acetate 1 M/methanol (solvent A), acetonitrile (solvent B), and reagent alcohol (solvent C) at a flow rate of

1.25 mL/min. Column temperature was kept at 25 °C. Table 1 shows the gradient scheme for eluting the pigments.

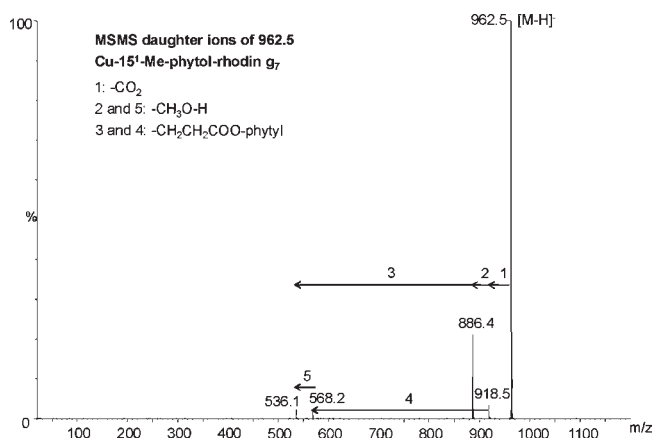
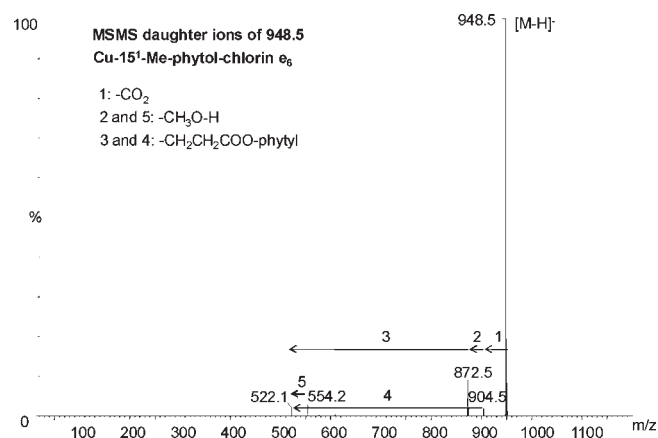
Mass spectrometric studies were performed on a Quadrupole/time-of-flight mass spectrometer (QToF Premier, Micromass Limited, Manchester, U.K.) equipped with an atmospheric pressure chemical ionization interface (APCI). Positive ion APCI conditions for chlorophyll analyses were: 30 μA corona current, collision energy 8 eV, cone voltage 35 V, source temperature at 110 °C, 50 L/h of cone gas, and the flow of nitrogen desolvation gas was 400 L/h, at 450 °C. Daughter ions spectra were obtained by APCI negative using various collision energies (8–35 eV). Sodium formate solution was used to calibrate the mass spectrometer from *m/z* 100–1500 with mass accuracy of <2 ppm. Detection and tentative identification of all of the chlorophyll derivatives were accomplished using in-line electronic absorption spectra recorded every 1.2 nm from 200 to 800 nm with a Waters model 996-PDA (Milford, MA). Quantification of all of the chlorophyll derivatives was based on PDA data from calibration curves with external standards at the respective λ_{max} of each compound.

Color Measurement. The color analysis has been made with a Minolta colorimeter (CR-300) using CIE *L*a*b** scale with illuminants C.

Table 3. Spectroscopic and Chromatographic Information of Pigments Quantified by HPLC–MS and Identified in the Samples^a

peak no.	pigment	K'_c	spectral data in the eluent					published data					solvent	ref		
			maxima (nm)				peak ratio	calcd ^c		obs ^d	maxima (nm)				peak ratio	
			I	II	III			$[M + H]^+$	$[M + H]^+$		I	II				III
1	pheophorbide <i>a</i>	2.05	410 ^b		666	1.94	593.276	593.277	409	666	1.85	MP	23			
2	Cu-pheophorbide <i>a</i>	2.76	400	425 ^b	651	1.10	654.190	654.193	400	424 ^b	654	1.00	MP	34		
3	pyropheophorbide <i>a</i>	3.27	410 ^b		666	2.03	535.270	535.272	409		666	1.85	MP	11		
4	Cu-pyropheophorbide <i>a</i>	4.63	400	425 ^b	651	1.10	596.184	596.182	404	422 ^b	653		MP	27		
5	unknown	9.14	447 ^b	577	626	10.98										
6	15 ² -Me-phytol-rhodin <i>g</i> ₇ ester	9.60	427 ^b	561	649	8.65	903.563	903.565	429 ^b		656		MP	21 ^e		
7	Cu-15 ² -Me-phytol-rhodin <i>g</i> ₇ ester	9.80	429 ^b	565	613	6.54	964.477	964.475	430 ^b		614		MP	21 ^e		
8	chlorophyll <i>b</i>	10.10	464 ^b	598	649	2.52	907.522	907.520	453 ^b	593	642	2.89	DE	35		
9	15 ² -Me-phytol-chlorin <i>e</i> ₆ ester	10.20	400 ^b		662	3.90	889.584	889.586	404 ^b	642	660		MP	21 ^e		
10	chlorophyll <i>b'</i>	10.35	464 ^b	598	649	2.52	907.522	907.520	453 ^b	593	642	2.89	DE	35		
11	Cu-15 ² -Me-phytol-chlorin <i>e</i> ₆ ester	10.65	407 ^b	504	629	2.46	950.498	950.495	408 ^b		635		MP	21 ^e		
12	chlorophyll <i>a</i>	10.95	432 ^b		662	1.14	893.543	893.545	430 ^b	615	661	1.32	DE	35		
13	chlorophyll <i>a'</i>	11.29	432 ^b		662	1.14	893.543	893.545	430 ^b	615	661	1.32	DE	35		
14	pheophytin <i>b</i>	11.73	437 ^b		651	4.59	885.552	885.550	433 ^b	599	654	4.81	DE	35		
15	Cu-pheophytin <i>b</i>	11.97	445 ^b		633	2.52	946.466	946.469	443 ^b		633	2.38	MP	34		
16	pheophytin <i>b'</i>	12.01	437 ^b		651	4.59	885.552	885.550	433 ^b	599	654	4.81	DE	35		
17	pyropheophytin <i>b</i>	12.35	437 ^b		651	4.34	827.547	827.549	435 ^b		654	3.83	MP	34		
18	pheophytin <i>a</i>	12.97	410 ^b	505	666	2.10	871.573	871.575	408 ^b	503	667	2.14	DE	35		
19	Cu-pyropheophytin <i>b</i>	13.12	444 ^b		633	2.02	888.461	888.463	443 ^b		633	2.38	MP	34		
20	15 ² -Me-phytol-isochlorin <i>e</i> ₄	13.27	400 ^b		667	2.94	845.594	845.595	399 ^b		662	2.92	MP	27 ^f		
21	pheophytin <i>a'</i>	13.39	410 ^b	505	666	2.10	871.573	871.575	408 ^b	503	667	2.14	DE	35		
22	Cu-pheophytin <i>a</i>	13.91	400	425 ^b	651	0.97	932.487	932.489	400	424 ^b	654	1.00	MP	34		
23	Cu-pheophytin <i>a'</i>	14.57	400	425 ^b	651	0.97	932.487	932.489	400	424 ^b	654	1.00	MP	34		
24	pyropheophytin <i>a</i>	14.63	410 ^b	505	666	1.97	813.568	813.565	409 ^b		666	1.85	MP	34		
25	Cu-15 ² -Me-phytol-isochlorin <i>e</i> ₄	15.50	408 ^b		630	2.34	906.508	906.510	406 ^b		630	2.55	MP	21 ^e		
26	Cu-pyropheophytin <i>a</i>	15.94	400	425 ^b	651	1.02	874.482	874.484	400	424 ^b	654	1.00	MP	34		

^a $K'_c = (t_r - t_m)/t_m$, where t_r is the retention time of the peak and t_m is the retention time of the unretained component. MP, mobile phase. DE, diethyl ether. ^b Soret band. ^c Calculated mass (the mass given is for the most abundant isotope, i.e., ⁶³Cu). ^d Observed mass, average Δ ppm < 2.5. ^e Compared to a similar structure without phytol. ^f Compared to formylpheophytin *a*.

Figure 3. MS/MS daughter ions of Cu-15²-Me-phytol-rhodin *g*₇ ester.Figure 4. MS/MS daughter ions of Cu-15²-Me-phytol-chlorin *e*₆ ester.

Statistical Analysis. Data were analyzed for differences between means using one-way analysis of variance (ANOVA). The Brown and Forsythe test¹⁹ was used as a post hoc comparison of statistical significance (p values < 0.05) using Statistica 6.0 (StatSoft, Inc., 2001).

RESULTS AND DISCUSSION

As color is the variable that should distinguish bright green table olives from regular table olives with a yellow-brown olive color, a Minolta colorimeter using CIE Lab scale was used.

Table 4. MS/MS Fragmentation Pattern of Chlorophyll Derivatives

pigment	$(m/z)^a$		MS/MS (m/z)
	calculated ^b	observed	
15 ² -Me-phytol-rhodin g_7 ester	901.5 a	901.5	857.6, 825.6, 507.2, 475.2
15 ² -Me-phytol-chlorin e_6 ester	887.6 a	887.6	843.6, 811.6, 493.2, 461.2
Cu-15 ² -Me-phytol-rhodin g_7 ester	962.5 a	962.5	918.5, 886.4, 568.2, 536.1
Cu-15 ² -Me-phytol-chlorin e_6 ester	948.5 a	948.5	904.5, 872.5, 554.2, 522.1
Cu-pheophytin b	945.5 b	945.5	662.2, 590.1
Cu-pyropheophytin b	887.5 b	887.5	608.1, 564.2, 536.1
Cu-pheophytin a	931.5 b	931.5	652.2, 580.2, 576.2, 565.1
Cu-pheophytin a'	931.5 b	931.5	652.2, 580.2, 576.2, 565.1
Cu-pyropheophytin a	873.5 b	873.5	594.2, 550.2, 522.2

^a $[M - H]^-$ is labeled with "a" and $[M]^+$ is labeled with "b". ^b The mass given is for the most abundant isotope, that is, ⁶³Cu.

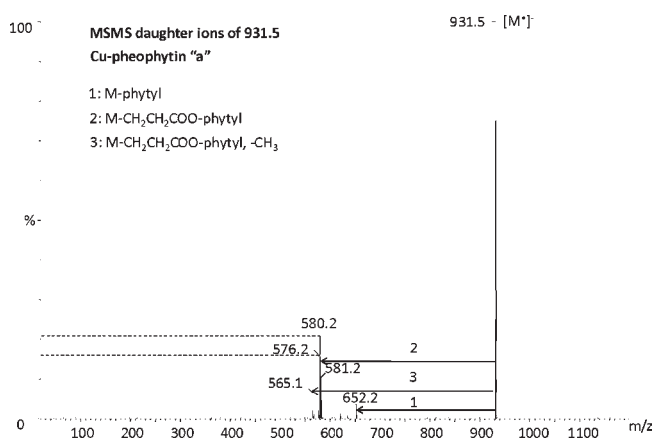
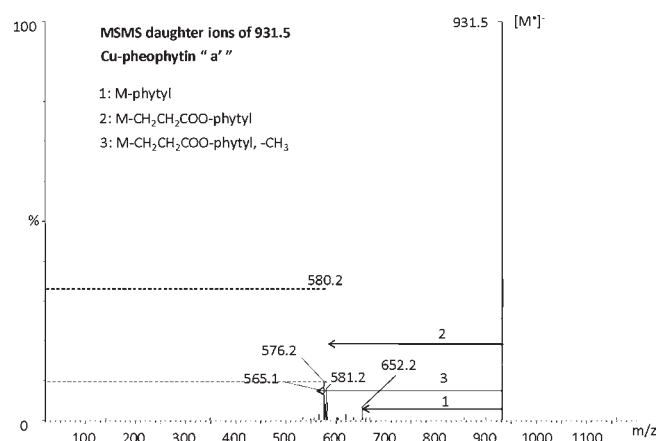
Figure 5. MS/MS daughter ions of Cu-pheophytin a .

Figure 1 displays $L^*a^*b^*$ values of all of the samples showing that bright green table olives are statistically different (p values < 0.05) from the regular table olives with all of the coordinates (-8.4 ± 2.6 vs -2.6 ± 0.3), with coordinate a^* (a^* , negative values indicate green, while positive values indicate magenta) being the most remarkable. In addition, there are no statistical differences (p values < 0.05) in $L^*a^*b^*$ coordinates within the samples of the two sets.

Once it was established that color was a determinant variable distinguishing the two sets of samples, the chemical compounds responsible for the color difference were identified and quantified by HPLC-MS/MS. Thus, Figure 2 shows the HPLC chromatograms, recorded at 650 nm, of chlorophylls, chlorophyll derivatives, and metallo-chlorophylls complexes (Table 2) determined in the five samples studied. Table 3 lists all of the pigments identified in Figure 2 with their chromatographic, spectroscopic, and mass characteristics. The information from the spectral data agrees basically with the published information. Although many of these pigments observed herein have been identified^{20–22} by mass spectrometry, nine pigment components (peaks numbered 6, 7, 9, 11, 15, 19, 22, 23, and 26) were not yet characterized by MS/MS.

Pigment profiles presented in Figure 2 show that bright green table olive samples (A, Castelvtrano-Mezzetta; B, Vincenzo's; C, Castelvtrano-WF) can be easily distinguished from Spanish table olives (D, *var.* Gordal; E, *var.* Manzanilla).

Figure 6. MS/MS daughter ions of Cu-pheophytin a' .

The chromatograms of the samples coded A–C showed the highest concentrations of Cu-15²-Me-phytol-chlorin e_6 ester (peak 11), Cu-pheophytin a (peaks 22–23), Cu-pyropheophytin a (peak 26), and chlorophylls a (peaks 12–13) and b (peaks 8 and 10).

Concerning the other two samples (coded D,E), the more representative compounds are pheophytin a (peaks 18 and 21) and b (peaks 14 and 16), 15²-Me-phytol-rhodin g_7 ester (peak 6), and 15²-Me-phytol-chlorin e_6 ester (peak 9), although minor pigments, such as pheophorbide a (peak 1) and pyropheophorbide a (peak 3), are also present. The most characteristic of the chlorophyll pigments in the Spanish style olive, processed by fermentation, is that all of the chlorophyll pigments have lost magnesium within the porphyrin ring, resulting in table olives with a yellow-brown olive color.

However, allomerized chlorophyll compounds detected in the Spanish style of table olives were identified primarily as phytol-chlorin e_6 and phytol-rhodin g_7 from their chromatographic characteristics and absorption spectral data.⁹ Subsequently, the nominal mass molecular ion obtained by MS led to its identification as 15-glyoxylic acid pheophytin a and 15-glyoxylic acid pheophytin b , compounds with similar chromatographic and spectral characteristics but different molecular mass.⁸ Finally, accurate mass MS/MS data from the ion fragmentation obtained in this work suggested a new structure such as 15²-Me-phytol-rhodin g_7 ester (peak 6) and 15²-Me-phytol-chlorin e_6 ester (peak 9).

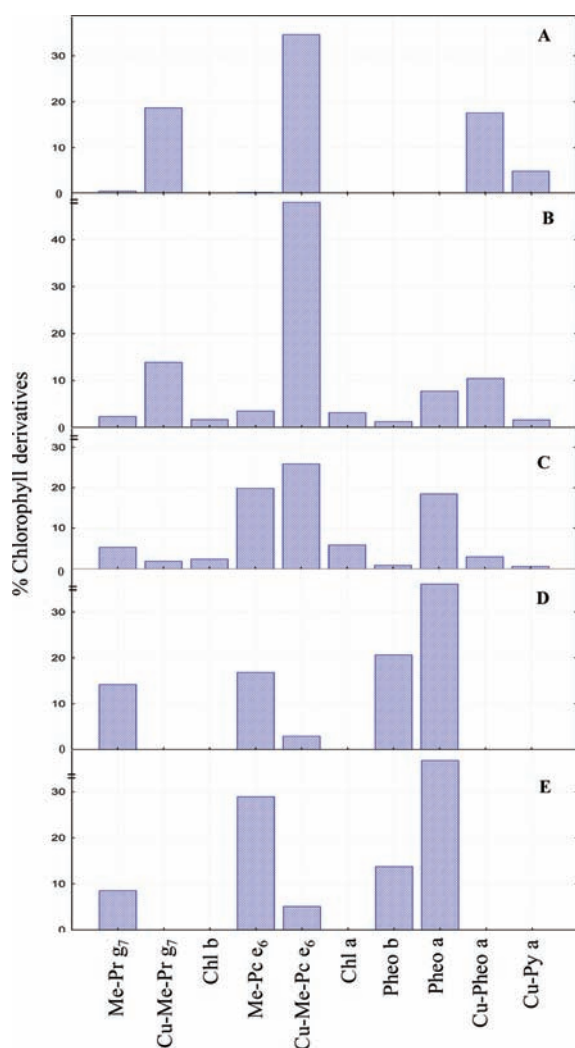


Figure 7. Percent of major pigments present in the five samples, coded A–E. Note: Me-Pr g_7 , 15^2 -Me-phytyl-rhodin g_7 ester; Cu-Me-Pr g_7 , Cu- 15^2 -Me-phytyl-rhodin g_7 ester; chl b , chlorophyll b ; Me-Pc e_6 ester, 15^2 -Me-phytyl-chlorin e_6 ester; Cu-Me-Pc e_6 , Cu- 15^2 -Me-phytyl-chlorin e_6 ester; chl a , chlorophyll a ; Pheo b , pheophytin b ; Pheo a , pheophytin a ; Cu-pheo a , Cu-pheophytin a ; Cu-py a , Cu-pyropheophytin a .

These pigments could be obtained by solvolysis of the isocyclic ring of the respective pheophytin according to the reaction scheme given by Hyninen.²³

From the set of newly identified pigments, the most abundant in bright green table olives were Cu- 15^2 -Me-phytyl-rhodin g_7 ester, Cu- 15^2 -Me-phytyl-chlorin e_6 ester, Cu-pheophytin a , and Cu-pyropheophytin a , and also 15^2 -Me-phytyl-rhodin g_7 ester and 15^2 -Me-phytyl-chlorin e_6 ester in the Spanish table olives. The MS/MS fragmentation patterns of the first two pigments, using a ramped collision energy, revealed the following daughter ions (Figures 3 and 4): m/z 918.5, 886.4, 568.2, and 536.1 for Cu- 15^2 -Me-phytyl-rhodin g_7 ester ($[M - H]^- = 964.5$) and 904.5, 872.5, 554.2, and 522.1 for Cu- 15^2 -Me-phytyl-chlorin e_6 ester ($[M - H]^- = 948.5$). The ions 918.5 and 904.4 are consistent with the loss of a carboxylic group ($M - CO_2$) from the respective molecular ion, while the loss of a methoxy group ($M - CH_3O - H$) from ions 918.5 and 904.4 would yield ions with m/z 886.4 and 872.5, respectively. Similarly, the loss of the phytol

ester chain ($M - CH_2CH_2COO$ -phytyl) from 886.4, 872.5, 918.5, and 904.4 would yield ions m/z 536.1, 522.1, 568.2, and 554.2, respectively. These pigments have been identified before as Cu- 15^2 -glyoxilic acid pheophytins a and b , respectively.¹¹ However, these pigments differ from the new structures identified herein due to a glyoxilic acid ($-COCOOH$) in R_4 (Table 4). No fragment such as $M - CO$ or $M - COCOOH$ has been found for the new structures, allowing the possibility of phytol-rhodin or phytol-chlorin structures.

The MS/MS fragmentation patterns of Cu-pheophytin a and its epimer a' using a fixed collision energy of 30 eV are shown in Figures 5 and 6. Both epimers yielded the same fragmentation patterns: the loss of phytol yielding the fragment 652.2 ($M - phytol$), the loss of the phytol ester chain yielding the mass 580.2 ($M - CH_2CH_2COO$ -phytyl), the loss of the phytol chain ($M - O$ -phytyl) plus the loss of the methoxy carbonyl group ($M - COOCH_3$) of R_4 yields the mass 576.2, and the loss of the methyl group from the methoxycarbonyl group of R_4 yields the mass 565.1. However, the abundance of fragment ions is different for Cu-pheophytin a and its respective epimer under identical conditions. A similar observation has been reported previously with pheophytins a and b .²⁰ The Cu-pheophytin a epimer exhibited an abundant fragment of 580.2 ($M - CH_2CH_2COO$ -Phytol), approximately 10% greater than for a , whereas the fragment m/z 576.2 occurred with minor intensity.

Table 4 shows MS/MS fragmentation patterns of 15^2 -Me-phytyl-rhodin g_7 ester and 15^2 -Me-phytyl-chlorin e_6 ester using fixed collision energy of 15 eV. The ions with m/z 857.6 and 843.6 represent the loss of a carboxylic group from the respective molecular ion, while the loss of a methoxy group from ions 857.6 and 843.6 yields the ions 825.5 and 811.6, respectively. The loss of the phytol ester chain from 825.5, 811.6, 857.6, and 843.6 yields the ions 475.2, 461.2, 507.2, and 493.2, respectively. MS/MS fragmentation patterns of Cu-pyropheophytin a used a fixed collision energy of 30 eV. The loss of phytol yielded the fragment 594.2 ($M - phytol$), the loss of the carboxy phytol yielded the mass 550.2 ($M - COO$ -phytyl), and the loss of the phytol ester chain yielded the mass 522.2 ($M - CH_2CH_2COO$ -phytyl). Less relevant due to their low concentration in bright green table olives are Cu-pheophytin b and Cu-pyropheophytin b . The MS/MS fragmentation pattern of Cu-pheophytin b is 945.5 and of Cu-pyropheophytin b is 887.5. The main daughter fragments obtained for Cu-pheophytin b (Table 4) are 666.2 ($M - phytol$) and 590.1 due to the loss of $-COO$ -phytyl and $-COOCH_3$ of the R_4 , while the main fragments obtained for Cu-pyropheophytin b (Table 4) are 608.1 ($M - phytol$), 564.2 ($M - COO$ -phytyl), and 536.1 ($M - CH_2CH_2COO$ -phytyl).

In regard to 15^2 -Me-phytyl-isochlorin e_4 and Cu- 15^2 -Me-phytyl-isochlorin e_4 , these compounds were identified previously as formylpheophytin a and its Cu derivative by their respective molecular ions.^{11,27} However, both mass accurate m/z values obtained herein are consistent with a structure with a $-CH_2COOCH_3$ group in R_4 and $-H$ in R_5 (15^2 -Me-phytyl-isochlorin e_4 and Cu- 15^2 -Me-phytyl-isochlorin e_4 , respectively) or with an ethyl group ($-CH_2CH_3$) and a carboxylic group ($-COOH$) in R_5 (15^2 -Et-phytyl-chlorin e_4 and Cu- 15^2 -Et-phytyl-chlorin e_4 , respectively) (Table 4). The two first options (15^2 -Me-phytyl-isochlorin e_4 and Cu- 15^2 -Me-phytyl-isochlorin e_4) are more likely to occur from the loss of the carboxylic group of the respective chlorin e_6 . Nevertheless, we could not acquire an appropriate MS/MS fragmentation pattern for these pigments that gave us a confident identification. Further MS/MS characterization is needed.

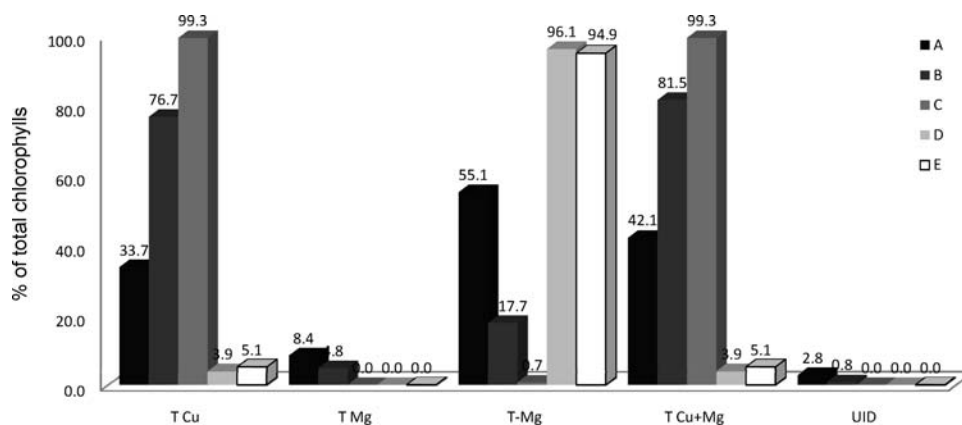


Figure 8. Percents of total Cu-metallo–chlorophylls (T Cu), total Mg-metallo–chlorophylls (T Mg), total chlorophylls without magnesium (T-Mg), and total Cu-metallo–chlorophylls plus Mg-metallo–chlorophylls (T Cu+Mg) and the unknown peak (UID) for each sample studied.

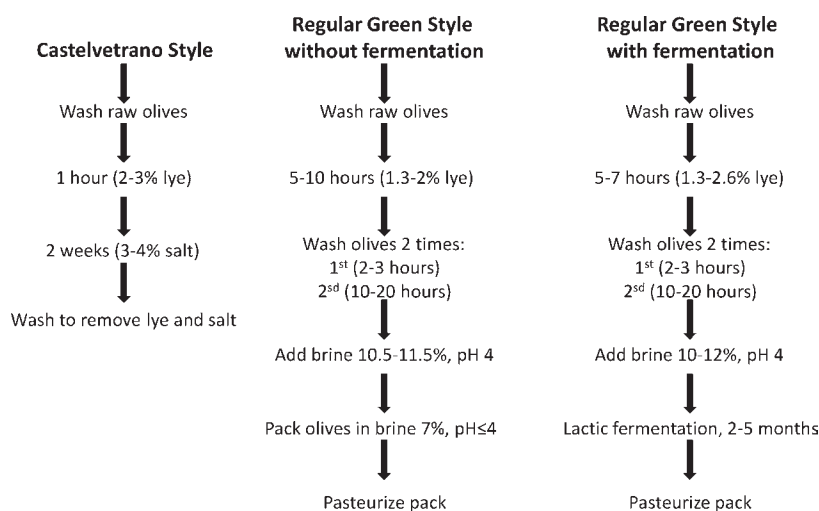


Figure 9. Scheme of the three different processing styles for table olives. Note: Source, refs 24, 25.

The quantitative analysis of the pigments present in the table olive samples (Figure 7) shows that the range of concentrations of the major pigments of bright green table olives were Cu-15²-Me-phytol-chlorin *e*₆ ester (26–48%), Cu-15²-Me-phytol-rhodin *g*₇ ester (2–18%), Cu-pheophytin *a* (3–18%), pheophytin *a* (0–18%), Cu-pyrropheophytin *a* (1–5%), chlorophyll *a* (0–6%), 15²-Me-phytol-rhodin *g*₇ ester (1–2%), and chlorophyll *b* (0–2%). The major pigments present in the Spanish table olives, in terms of concentration, were pheophytin *a* (36–37%), pheophytin *b* (13–20%), 15²-Me-phytol-chlorin *e*₆ ester (17–29%), and 15²-Me-phytol-rhodin *g*₇ ester (8–15%).

The relative amount of various chlorophyll derivatives, with or without metal within the porphyrin ring, allows differentiation among the samples. Thus, Figure 8 shows the percent of Cu-metallo–chlorophylls (T Cu), Mg-metallo–chlorophylls (T Mg), chlorophylls without magnesium (T-Mg), Cu-metallo–chlorophylls plus Mg-metallo–chlorophylls (T Cu+Mg), and an unknown compound (UID). From this comparison, we have observed that bright green table olives contain 33–99% of Cu-metallo–chlorophyll complexes, while Spanish table olives have approximately 95% of chlorophyll derivatives without the magnesium porphyrin complex and approximately 5% of Cu–porphyrin complex. Furthermore,

bright green table olives contain small quantities of Mg-metallo–chlorophylls (0–8% due to chlorophylls *a* and *b*) in contrast to Spanish table olives that do not contain detectable levels of these compounds. The differences may be attributed to the different methods of the industrial processing of table olives (Figure 9). The Castelvetro style (samples coded A and C) is manufactured without fermentation and requires approximately 2 weeks for processing, although the shelf life of its table olives is only a few weeks under warm storage conditions.²⁴ The presence of Mg-metallo–chlorophylls (5–8%) in samples B and C implies that the processing style was Castelvetro. Sample A, however, does not contain Mg-metallo–chlorophylls, which can be due to a modification of the Castelvetro processing style to prolong the shelf life such as pasteurization. In contrast, the Spanish style olive, based on a lactic fermentation process (Figure 9), requires 2–5 months^{24,25} and results in the disappearance of chlorophylls *a* and *b* in less than 120 days.²⁶

Previous researchers^{10–12,27,28} have reported a GS alteration of the Gordal variety of table olives where the color alteration is due to Cu-metallo–chlorophylls derivatives. One report shows the percent of total Cu-metallo–chlorophylls *a* with respect to the total of *a* series after the lactic fermentation with amounts

ranging from 2% to 11%.²⁷ These data are lower than our observation for three commercially available bright green table olive samples (36.1–99.8%).

We have found that the total elemental Cu from the quantification of Cu-metallo–chlorophyll complexes present in the three bright green table olive samples varies between 1.3 mg and 4.7 mg/kg, and within the range of mineral Cu (1.7–11.0 mg/kg) quantified in different varietal table olives²⁹ and fresh olives (3.5–7.2 mg/kg).³⁰ Thus, there appears to be adequate quantities of Cu native to the fruit to allow for regreening to occur.¹⁰

The copper absorbed by the olive fruit could arise from copper in the soil but most likely derives from the content of copper in the pesticides applied to prevent soapy olive (fungus infection by *Gloeosporium olivarum*) and “repilo” (fungus infection by *Cycloconium oleaginum*).

As such, the bright green color of table olives is mostly due to the existence of Cu within porphyrin ring, but the presence of Cu-metallo–chlorophylls has not been reported in fresh olives, fruits, or leaves. However, these compounds can be generated using copper salts (CuCl₂, CuSO₄) during the industrial processing of food^{4–7} or generated during the commercial processing of table olives with endogenous copper absorbed by the fruit.²⁷ We assume that the Cu–chlorophyll compounds are produced without adding copper salts and that the industrial processing has been carried out according to international rules and regulations.^{31,32}

Olives with GS alteration might be a defect created by the partial formation of metallo–chlorophyll pigments during the manufacture of green table olives resulting in a color that is not uniform. Although color is important for consumer acceptance, the bright green table olives provide a source of metallo–chlorophyll derivatives (Cu-Me-phytol-chlorin e₆, 26–48%; and Cu-pheophytin a, 3–18%) that have been reported to possess an inhibitory activity against mutagenesis and higher antioxidant activity as well.³³ Further investigation is needed to understand the processing method for regreening table olives that will result in optimal uniformity in color and consumer acceptance.

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REFERENCES

- (1) Humphrey, A. M. Chlorophyll. *Food Chem.* **1980**, *5*, 57–67.
- (2) Jones, I. D.; White, R. C.; Gibbs, E.; Butler, L. S.; Nelson, L. A. Experimental formation of zinc and copper complexes of chlorophylls derivatives in vegetables tissue by thermal processing. *J. Agric. Food Chem.* **1977**, *25*, 149–143.
- (3) Von Elbe, J. H.; Huang, A. S.; Attoe, E. L.; Nank, W. K. Pigment composition and color of conventional Veri-Green canned beans. *J. Agric. Food Chem.* **1986**, *34*, 52–54.

- (4) Segner, W. P.; Ragusa, T. J.; Nank, W. K.; Hoyle, W. C. Process for the preservation of green color in canned vegetables. U.S. patent 4,473,591, 1984.
- (5) Hekal, I. M.; Erlandson, P. M. Coating and container for retention of green vegetables. U.S. patent 4,615,924, 1986.
- (6) Leake, L. H.; Kirk, L. K. Method for color preservation in canned vegetables. U.S. patent 5,114,724, 1992.
- (7) LaBorde, L. F.; von Elbe, J. H. Method for improving the color of containerized green vegetables. U.S. patent 5,482,727, 1996.
- (8) Minguez-Mosquera, M. I.; Gandul-Rojas, B.; Garrido-Fernandez, J. Preparation of Cu (II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography. *J. Chromatogr. A* **1996**, *731*, 261–271.
- (9) Minguez-Mosquera, M. I.; Gallardo-Guerrero, L. Anomalous transformation of chloroplastic pigments in gordal variety olives during processing for table olives. *J. Food Prot.* **1995**, *58*, 1241–1248.
- (10) Minguez-Mosquera, M. I.; Gallardo-Guerrero, L.; Hornero-Mendez, D.; Garrido-Fernandez, J. Involvement of copper and zinc ions in green staining of table olives of the variety Gordal. *J. Food Prot.* **1995**, *58*, 564–569.
- (11) Gandul-Rojas, B.; Gallardo-Guerrero, L.; Minguez-Mosquera, M. I. Identification of oxidized chlorophylls and metallochlorophyll complexes of copper in table olives (var. Gordal) with green staining alteration. *J. Food Prot.* **1999**, *62*, 1172–1177.
- (12) Gallardo-Guerrero, L.; Hornero-Mendez, D.; Minguez-Mosquera, M. I. Pectin as possible source of the copper involved in the green staining alteration of cv. Gordal table olives. *J. Agric. Food Chem.* **2002**, *50*, 6746–6751.
- (13) Huang, S. C.; Hung, C. F.; Wu, W. B.; et al. Determination of chlorophylls and their derivatives in *Gynostemma pentaphyllum* Makino by liquid chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **2008**, *48*, 105–112.
- (14) Pickering, M. D.; Keely, B. J. Alkyl sulfur chlorophyll derivatives: Preparation and liquid chromatography-multistage tandem mass spectrometric characterisation of analogues of naturally occurring sedimentary species. *Org. Geochem.* **2007**, *39*, 1046–1050.
- (15) Egner, P. A.; Stansbury, K. H.; Snyder, E. P.; et al. Identification and characterization of chlorin e(4) ethyl ester in sera of individuals participating in the chlorophyllin chemoprevention trial. *Chem. Res. Toxicol.* **2000**, *13*, 900–906.
- (16) Mendes-Pinto, M. M.; Ferreira, A. C. S.; Caris-Veyrat, C.; et al. Carotenoid, chlorophyll, and chlorophyll-derived compounds in grapes and Port wines. *J. Agric. Food Chem.* **2005**, *53*, 10034–10041.
- (17) Sievers, G.; Hynninen, P. H. Thin-layer chromatography of chlorophylls and their derivatives on cellulose layers. *J. Chromatogr.* **1977**, *134*, 359.
- (18) Minguez-Mosquera, M. I.; Garrido-Fernández, J. Chlorophyll and carotenoid presence in olive fruit (*Olea Europaea* L.). *J. Agric. Food Chem.* **1989**, *37*, 1–7.
- (19) Brown, M. B.; Forsythe, A. B. Robust tests for the equality of variances. *J. Am. Stat. Assoc.* **1974**, *69*, 364–367.
- (20) Gauthier-Jaques, A.; Borltlik, K.; Hau, J.; Fay, L. B. Improved method to track chlorophyll degradation. *J. Agric. Food Chem.* **2001**, *49*, 1117–1122.
- (21) Mortensen, A.; Geppel, A. HPLC–MS analysis of green food colorant sodium copper chlorophyllin. *Innovative Food Sci. Emerging Technol.* **2007**, *8*, 419–425.
- (22) Van Breemen, R. B.; Canjura, F. L.; Schwartz, S. J. Identification of chlorophyll derivatives by mass spectrometry. *J. Agric. Food Chem.* **1991**, *39*, 1452–1456.
- (23) Hynninen, P. H. Chlorophylls. IV. Preparation and purification of some derivatives of chlorophylls a and b. *Acta Chem. Scand.* **1973**, *27*, 1771–1780.
- (24) Kailis, S. G.; Harris, D. *Producing Table Olives*; Edit Landlinks Press: Collingwood VIC (Victoria), Australia, 2007; ISBN 9780643092037.
- (25) Garrido Fernández, A.; Fernández Díez, M. J.; Adams, M. R. *Table Olives: Production and Processing. Technology & Engineering*; Springer: New York, 1997; ISBN 0412718103.

(26) Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Mínguez-Mosquera, J. Mechanism and kinetics of the degradation of chlorophylls during the processing of green table olives. *J. Agric. Food Chem.* **1994**, *42*, 1089–1095.

(27) Gallardo-Guerrero, L.; Mínguez-Mosquera, M. I.; Gandul-Rojas, B. Physicochemical conditions modulating the pigment profile in fresh fruit (*Olea europea* var. Gordal) and favoring interaction between oxidized chlorophylls and endogenous Cu. *J. Agric. Food Chem.* **2007**, *55*, 1823–1831.

(28) Gallardo-Guerrero, L.; Milicua, J. C. G.; Salvador, A. M.; Jarengalan, M.; Mínguez-Mosquera, M. I. Pigment-lipoprotein complexes in table olives (Cv. Gordal) with green staining alteration. *J. Agric. Food Chem.* **2003**, *51*, 1724–1727.

(29) Lopez, A.; Garcia, P.; Garrido, A. Multivariate characterization of table olives according to their mineral nutrient composition. *Food Chem.* **2008**, *106*, 369–378.

(30) Biricik, G. F.; Basoglu, F. Determination of mineral contents in some olives (Samanli, Domat, Manzanilla, Ascolana) varieties. *Gida* **2006**, *3*, 67–75.

(31) International Olive Oil Council (IOOC). Trade standards applying to table olives. COI/OT/NC No. 1., Madrid, Spain, 2004.

(32) IOOC/CODEX. Codex standard for table olives (CODEX STAN 66-1981), 1981.

(33) Ferruzzi, M. G.; Bohm, V.; Courtney, P. D.; Schwartz, S. J. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *Food Chem. Toxicol.* **2002**, *67*, 2589–2595.

(34) Roca, M.; Mínguez-Mosquera, M. I.; Gallardo-Guerrero, L.; Gandul-Rojas, B. Control of olive oil adulteration with copper-chlorophyll derivatives. *J. Agric. Food Chem.* **2010**, *58*, 51–56.

(35) Hynninen, P. H.; Ellfolk, N. Chlorophylls. I. Separation and isolation of chlorophylls a and b by multiple liquid–liquid partition. *Acta Chem. Scand.* **1973**, *27*, 1463–1477.