

INVESTIGATIONS OF COMMERCIAL SODIUM COPPER CHLOROPHYLLIN DEGRADATION AFTER ULTRAVIOLET IRRADIATION

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(ORIGINAL SCIENTIFIC PAPER)

UDC: 547.979.7:614.875

DOI: 10.5937/savteh2302042P

Chlorophyllins are naturally derived colors that provide green hues to food products in oil- and water-based systems. Due to their good antimicrobial activity, in order not to contaminate the surface of food products and to increase product expiration dates and safety, the use of commercial chlorophyllin is common in the form of antimicrobial films that could cover fresh or processed food. UV irradiation effects on commercial sodium copper chlorophyllin are examined to estimate the potential application risk of this chlorophyll derivative for use in foods and cosmetics. Photodegradation of an aqueous 1×10^{-4} M commercial sodium copper chlorophyllin solution was monitored after ultraviolet irradiation. The samples were irradiated for a period of 0 - 60 min. in a cylindrical photochemical reactor called "Rayonnet" with symmetrically placed lamps that emit an energy flux of 12 W/m^2 at 300 nm. Ultra-high liquid chromatography coupled with a diode array and electrospray ionization mass spectrometry was used to monitor the loss of major components of sodium copper chlorophyllin after irradiation, as well as, to detect possible new products. It is estimated that the photodegradation of all detected compounds follows first-order kinetics. UHPLC analysis showed the loss of the major chlorophyllin component, copper chlorin e_6 , at a rate faster than the overall detected components, in the range of $7.02 \times 10^{-2} \text{ min}^{-1}$. The observed photosensitivity of sodium copper chlorophyllin was lower than to the natural chlorophylls, and raising the possibility of its use in food and cosmetics. The main detected photoproducts are oxidized compounds of chlorophyllin. For all these reasons, the rapid loss of copper chlorin e_6 , may reduce some of the main commercial sodium copper chlorophyllin dietary benefits, like antimutagenic and antioxidant activity.

Keywords: chlorophyllin, E141, degradation, irradiation, UHPLC-DAD-ESI-MS

Introduction

Sodium copper chlorophyllin (CHLIN) is known as chlorine with four closed-chain pyrroles and a fifth isocyclic five-membered ring (Figure 1). It is a chlorophyll derivative, known as a green pigment, that is widely used in many industries, not only because of its color but also because of many biological activities [1]. Due to some limitations in light, high temperatures, oxygen, the propensity to form aggregates, and its insolubility in water, chlorophyll typically has no practical use in products [2-4]. Opposite to chlorophyll, CHLIN structurally does not contain a wicker chain ($\text{C}_{20}\text{H}_{39}\text{O}$ - in position C-17, Figure 1), which gives it hydrophilicity and higher stability [5]. As a water-soluble form, the use of CHLIN in food, cosmetics, and other commercial products is more often compared to chlorophyll.

CHLIN is usually produced in the chlorophyll saponification reaction and its coppering occurs at the end. This

reaction produced a complex compound mixture [6]. Commercial CHLIN represents a mixture of several chlorine compounds: copper chlorin e_4 , copper chlorin e_6 , and often pheophorbides *a* and *b*, as well as the corresponding copper pheophorbides (Figure 1).

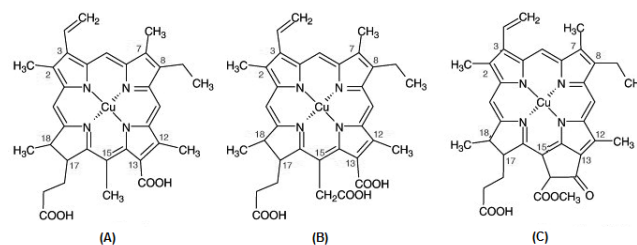


Figure 1. Structure of the main CHLIN mixture components: (A) copper chlorin e_4 , (B) copper chlorin e_6 , (C) copper pheophorbide *a*.

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Received: 03.10.2023.

Accepted: 23.10.2023.

CHLIN is known as a dietary supplement as well because of its obvious chemopreventive activity. Due to their antimicrobial activity [7-9], many authors have suggested the use of CHLIN and its derivatives as antimicrobial films that could cover fresh or processed food products. This would prevent food surface contamination while increasing food expiration date and safety [9]. In addition, for a better appearance of food and easier application, the most common CHLIN used form are gels [10]. The antimicrobial efficiency was noticed at $1.5 \times 10^{-5} - 5 \times 10^{-3}$ M CHLIN concentrations [11]. As a food-grade color (E141), it is used in ice creams, dry beverage mixes, icing, and confectionery. In the field of agriculture, this pigment is used as a pesticide to save the crops from different kinds of bugs or pesticides that attack the crops in different seasons. CHLIN has been approved for use by the European Community. On the other hand, its use in the United States has been limited [12].

In previous investigations, CHLINS have been identified not only with good antioxidant but anticancer activity as well. Namely, CHLINS structures have shown the opportunity to complex with toxins and carcinogenic aromatic hydrocarbons [13,14]. CHLIN is distinguished by an intense Q-band at 630 nm [15], which is an ideal characteristic for use in photodynamic therapy (PDT), a technique applied in the case of cancer treatments and damaging diseased living tissue treatments with visible light in the presence of photosensitizers and O_2 [16,17]. The most common chlorophylls used in PDT are chlorine e_6 and other synthetic chlorines [18-20].

In cosmetics, CHLIN is used in many different products, in the treatment of premature skin aging, in the treatment of acne [21,22], oily skin, and enlarged pores [23,24], as a colorant for cosmetics, in shampoos, soaps, lotions, perfumes, and toothpaste. Oftentimes, CHLIN cosmetic products contain CHLIN incorporated into a carrier as well.

The stability of CHLIN depends on many factors, and because of that, the control of factors that affect its stability is a necessary prerequisite for the ability to control its function and activity. Since photosynthetic pigments primarily absorb in the visible spectra region, their composition is significantly photodegraded when exposed to *in vivo* and *in vitro* [25]. Copper chlorine e_6 has been shown to be less stable and, in some cases, degrades as a result of heat treatment during food production [26]. Also, its stability depends on the pH value of the environment. Copper chlorine e_4 , on the other hand, is relatively stable under these conditions and can be used as an indicator substance for further analysis [26]. The observed sensitivity of CHLIN is similar to that of natural chlorophyll and increases the possibility of discoloration when used in food products. Also, the implication of copper chlorine e_4 degradation may lead to a change in potential nutritional benefits, mostly antimutagenic and antioxidant activity [26].

CHLIN behavior and possible degradation product formation after irradiation are not completely investigat-

ed to evaluate its potential for further use. Ultrahigh liquid chromatography coupled with a diode array and electrospray ionization mass spectrometry was employed to analyze degradation product formation induced by continuous UV irradiation and to provide the data for kinetic analysis. The obtained data presented in this paper may serve for further CHLIN activity investigations in active formulations. Also, applied optimized UHPLC-DAD-ESI-MS can be characterized as rapid, sensitive, and selective, with great potential for use in further studies.

Materials and Methods

Commercially purified chlorophyllin was used for experiments in this paper: $C_{34}H_{31}CuN_4Na_3O_6$ - sodium copper chlorophyllin, TLC TOKYO CHEMICAL INDUSTRY CO. For the analysis, the aqueous solutions of chlorophyllin were used at concentrations of 1×10^{-4} M. Methanol, water, and MeCN (LC-MS grade) were purchased from Fisher Scientific Co. (USA).

UV irradiation

The irradiation of CHLIN samples was performed in a cylindrical photochemical reactor "Rayonnet", with eight lamps symmetrically placed. The emission irradiation maximum was at 300 nm. The total emitted measured energy flux measured by the UV-meter, Solarmeter, Mode I8.0 UV Meter, and Solartech, was 12 W/m^2 . CHLIN samples were irradiated in quartz cuvettes that were placed on a rotating circular holder in the reactor, at room temperature.

Ultra-high-performance liquid chromatography (UHPLC) diode array – electrospray ionization mass spectrometry analysis (ESI-MS)

UHPLC analysis was performed using a Dionex Ultimate 3000 UHPLC+ system equipped with a diode array (DAD) detector and Fleet Ion Trap Mass Spectrometer, Thermo Fisher Scientific, USA. The separations were conducted on a Hypersil gold C18 column ($50 \times 2.1 \text{ mm}$, $1.9 \mu\text{m}$) at an isocratic run with 15% v/v H_2O , 75% v/v MeOH, and 10% v/v MeCN at $t=25^\circ\text{C}$. The injection volume was $0.10 \mu\text{L}$. Absorption spectra were recorded on a DAD-detector (with a total range between 200 and 800 nm) at the detection wavelength, λ_{det} , 400 nm. MS analysis was performed using an LCQ 3D ion trap mass spectrometer with electrospray ionization in positive ion mode. The ESI-source parameters were: capillary voltage 50 V, tube lens voltage 90 V, capillary temperature 350°C , sheath and auxiliary gas flow (N_2) 32 and 8 (arbitrary units), respectively. MS-spectra were acquired by full range acquisition of m/z 100 to 1000. For fragmentation analysis, a data-dependent scan was employed by deploying collision-induced dissociation (CID). The normalized collision CID energy was set at 20 eV.

Identification of the major compounds in the not-irradiated CHLIN solution was provided by using the corresponding UV-Vis and MS/MS spectra, as well as the

elution order in the chromatograms in comparison to the ones found in the literature. The peak areas in the obtained UHPLC-DAD chromatograms at 400 nm were used for the kinetic analysis of the major CHLIN compound degradation induced by continual UV-B irradiation. CHLIN modifications, *i.e.*, formation of UV-B irradiation degradation products were followed by the Vis spectra of the DAD signals and the MS spectra with the corresponding molecular ion peaks ($[M+H]^+$), as well as characteristic ion fragmentation within selected peaks (MS/MS), from the corresponding chromatogram. For instrument control data acquisition and data analysis, Xcalibur software (version 2.1) was used.

Results and discussion

Chlorophyllins have been widely used for decades for medical purposes, food color, and as dietary supplements, but they are still not examined for their accurate composition. The traditional spectrophotometric methods are considered inaccurate and ineffective in identifying chlorine constituents because of their similar spectral characteristics [5]. According to previous investigations, commercial chlorophyllins in most cases possess two major chlorin components, copper chlorin e_4 and copper chlorin e_6 . Also commonly identified are chlorin ethyl esters [27].

The UHPLC-DAD-ESI-MS analysis used in this study reveals four primary peaks that correspond to CHLIN components by MS fragmentation ion peaks and corresponding spectral data (presented in Table 1): Pheophorbide *a*, Pheophorbide *b*, copper chlorin e_6 , and copper (iso)chlorin e_4 . After irradiation, a loss of color in the aqueous solution of CHLIN was observed. The degradation of CHLIN in water after 60 min of irradiation is more than 80%.

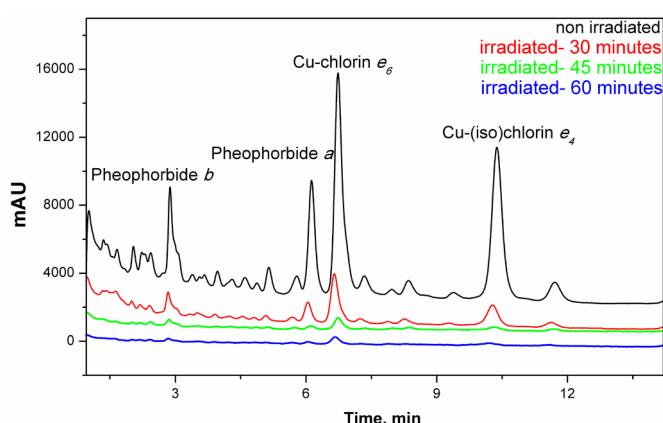


Figure 2. UHPLC-DAD chromatograms for not-irradiated and irradiated CHLIN, at 400 nm.

The results of UHPLC-DAD-ESI-MS have shown that all CHLIN components are degraded under UVB irradiation; degradation is higher at longer irradiation times (Figure 2). Peak assignments of chlorophyllins separated by UHPLC-chromatography before and after irradiation are

presented in Table 1.

Table 1. Peak assignment of CHLIN separated by UHPLC-chromatography.

Peak No.	t_{ret} , min (UHPL C-MS)	UV-Vis spectral data Soret, Q_y band, nm (Soret/ Q_y ratio)	Molecular ion peak $[M+H]^+$, m/z	Fragment ions, m/z	Assignments (References)	Degradation rate constant, k/min^{-1}	R^2
Not-irradiated CHLIN (elution order by McCook et al. [28])							
1	1.76	403*, -	597	579,569,55 1/553,524,496	Chlorin e_6 [29], [30]	-	-
2	1.80	-	553	509,494,408,355	Chlorin e_4 [5]	0.0377	0.99
3	3.03	435, 623 (3.4)	607	607,548,519,505,575,447	Pheophorbide <i>b</i> [31]	0.0440	0.97
4	5.60	-	593	593,575,531,491,458,413	Pheophorbide <i>a</i> [31]	0.0454	0.99
5	6.40	407, 635 (2.6)	659	644,614,540,514	Copper chlorin e_6 [6]	0.0702	0.98
6	7.10	406, 639 (2.5)	659	644,614,540,514	Copper (iso)chlorin e_6 [27]	0.0595	0.98
7	10.80	405, 627 (3)	613	555,540,511,494,481	Copper (iso)chlorin e_4 [5]	0.0797	0.97
UV-B irradiated CHLIN, proposed photooxidation products							
P1	1.17	403*, -	599	581,553,526,507	Product of chlorin e_6	30	
P2	7.00	404, 619 (2.6)	661	646,615,583,542,525,481,525	Product of copper chlorin e_6	30	
P3	7.43	402*, -	645	627,599,571,545,443	Product of copper (iso)chlorin e_4	60	

* - only Soret band is clearly visible in the UV-Vis spectrum

In addition to the observed irreversible degradation of CHLIN, the investigation revealed the creation of additional compounds, analysis also showed new product formation, most likely oxidative degradation products, which are marked as P1, P2, and P3. The ESI-MS/MS spectrum of the detected degradation compounds is assigned as an oxidation product of chlorin e_6 , copper chlorin e_6 , and copper (iso)chlorin e_4 and their elimination fragment possibilities are presented in Figures 3, 4, and 5, respectively. In Figures 3, 4, and 5 increment was shown in the proposed structure of the corresponding product molecule. The products (Figures 3, 4, and 5), of the CHLIN compounds were first detected after 30 min of the irradiation treatment (Table 1). The assignment was done by using similarity structures of the chlorins e_4 and e_6 and their copper derivatives with the structure of protoporphyrin IX, with vinyl and methyl groups in the positions C-2 and C-3, respectively [32], in the cyclic tetrapyrrole of the porphyrin molecule (Figure 1).

RT: 1.15-1.21

F: ITMS + c ESI d Full ms2 599.41@cid20.00 [155.00-1210.00]

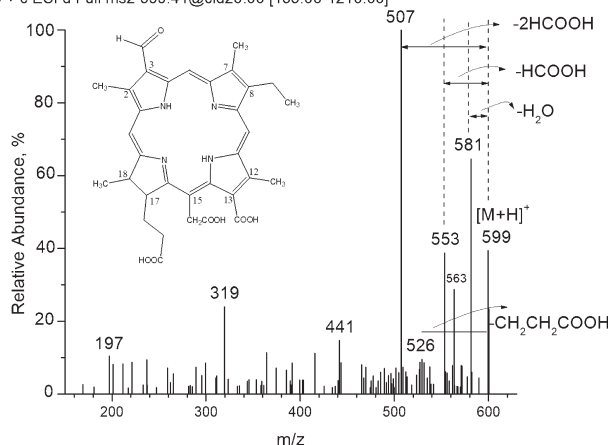


Figure 3. ESI-MS/MS spectrum of the compound P1 assigned as oxidation product of the chlorin e_6 and elimination fragments possibilities. In the increment was shown proposed structure of the corresponding product molecule, P1.

RT: 7.02

F: ITMS + c ESI d Full ms2 661.33@cid20.00 [170.00-800.00]

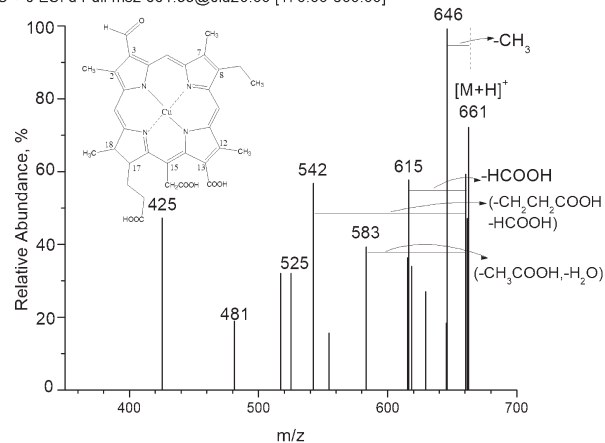


Figure 4. ESI-MS/MS spectrum of the compound P2 assigned as an oxidation product of the copper chlorin e_6 and elimination fragments possibilities. In the increment was shown proposed structure of the corresponding product molecule, P2.

RT: 7.43

F: ITMS + c ESI d Full ms2 645.34@cid20.00 [165.00-700.00]

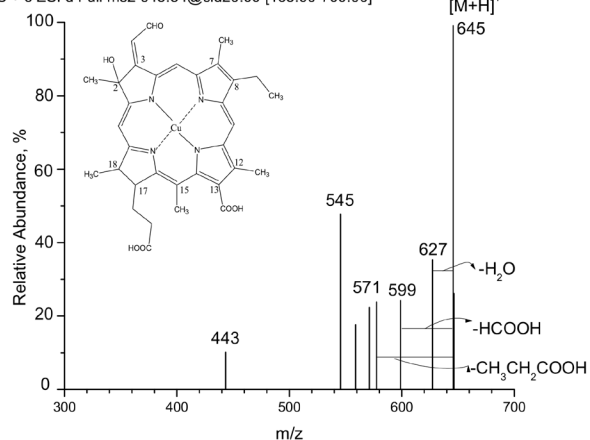


Figure 5. ESI-MS/MS spectrum of the compound P3 assigned as an oxidation product of the copper (iso)chlorin e_4 and elimination fragments possibilities. In the increment, the proposed structure of the corresponding product molecule, P3.

Namely, photoreactions of protoporphyrin IX and related porphyrins in the presence of molecular oxygen lead to the formation of several photo-(degradation) products with molecular masses of 2 and 32 units greater, as shown in the paper of Cox et al. [32]. A schematic preview of the proposed irradiation induced oxidation product formation pathway from the CHLIN compounds is shown in Figure 6.

Similarly, for the chlorin e_6 molecule, UV-B irradiation in the presence of oxygen from the air has given the possibility of oxidation and $-CH_2$ elimination in the molecule in the position of the vinyl group (C-3), leading to the formation of product P1 with the structure and MS/MS spectrum shown in Figure 3. The corresponding MS/MS spectrum of the P1 is similar to the one for chlorin e_6 : molecular ion peaks $[M+H]^+$ at m/z 597 (chlorin e_6) and m/z 599 (P1) have shown fragment ions obtained by elimination of 18 and 46/44 units, corresponding to H_2O and $HCOOH/CO_2$, which is appropriate spectral behavior for the selected structures ($-COOH$ groups in the positions C-13, 15, and 17, Figure 1). The products that are formed this way are unstable and susceptible to further photodestruction, having photosensitizing properties as well.

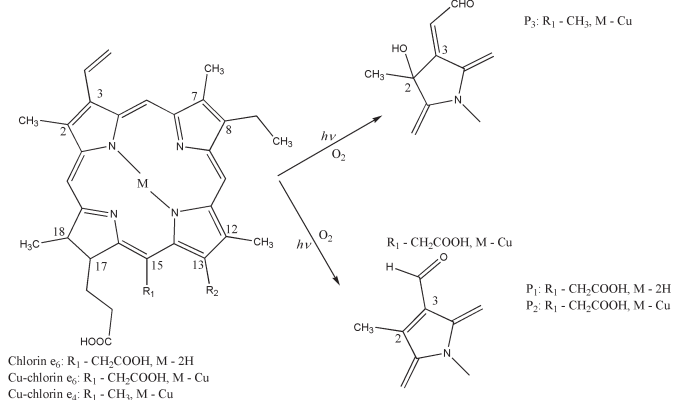


Figure 6. Proposed irradiation induced oxidation product formation from the CHLIN compounds, similar to the ones shown in the study of Cox et al. [32] from protoporphyrin IX.

Further analysis established the degradation kinetics of all detected CHLIN components. The UV-induced CHLIN degradation in the water solution follows the first-order kinetic model. Degradation constants indicate that CHLIN components do not degrade at the same rate (Table 1). The highest degradation constants were observed for copper chlorin e_6 and copper (iso)chlorin e_4 , 0.0702 and 0.0797 min^{-1} , respectively. In the investigation of Ryan-Stoneham and Tong [33], first-order kinetic model was also observed for the degradation of chlorophyll in peas under different pHs. The previous results for porphyrin-type compounds that indicate the first order kinetics are similar to those obtained in this paper [4, 34].

CHLIN has been shown to have a broad range of benefits, especially for human health. As the main CHLIN-detected compound with potential antioxidant activity, copper chlorin e_4 , and copper chlorin e_6 can reduce lipid peroxidation that diminishes food and food product quali-

ty [12], [35]. The irradiation instability of CHLIN observed in this paper indicates a loss of antioxidant activity as well. Also, the loss of intense green color after irradiation influences the visual quality of food where CHLIN is used.

Conclusions

The effects of irradiation with a wavelength of 300 nm, which corresponds to UVB rays, on CHLIN, were investigated using UHPLC-DAD-ESI-MS analysis. Applied optimized UHPLC-DAD-ESI-MS can be considered selective, rapid, and sensitive and may serve further studies.

The analysis detected four main peaks corresponding to CHLIN components: Pheophorbide *a*, Pheophorbide *b*, copper chlorin *e*₆, and copper (iso)chlorin *e*₄. Kinetic plots of degraded components fit the first-order kinetic model, and a higher degradation constant has been observed for copper chlorin *e*₆ and copper (iso)chlorin *e*₄.

The observed new degradation products, as well as the observed instability of the detected components of CHLIN indicate its instability under UV-B irradiation. Therefore, it is generally recommended to protect products containing CHLIN as an active component from the effects of light.

Acknowledgment

Authors wish to express their gratitude to the Republic of Serbia - Ministry of Education, Science and Technological Development, Program for financing scientific research work, number 451-03-47/2023-01/200133.

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Izvod

ISTRAŽIVANJA DEGRADACIJE KOMERCIJALNOG NATRIJUM-BAKAR HLOROFILINA NAKON ULTRALJUBIČASTOG ZRAČENJA

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UDK: 547.979.7:614.875
DOI: 10.5937/savteh2302042P

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Hlorofilini su boje prirodnog porekla koje daju zelene nijanse prehrambenim proizvodima u sistemima na bazi ulja i vode. Zbog dobre antimikrobne aktivnosti, kako bi se produžio rok trajanja i bezbednost prehrambenih proizvoda i izbegla kontaminacija na površini hrane, uobičajena je upotreba komercijalnog hlorofilina u vidu antimikrobnih filmova koji bi prekrivali hranu, svežu i prerađenu. Uticaj ultraljubičastog zračenja na komercijalni natrijum-bakar hlorofilin je proučavan da bi se procenio potencijalni rizik primene ovog derivata hlorofila za upotrebu u hrani i kozmetici. Fotodegradacija vodenog rastvora komercijalnog natrijum-bakar hlorofilina koncentracije 1×10^{-4} M, je praćena nakon dejstva ultraljubičastog zračenja. Uzorci su ozračeni u periodu od 0 - 60 min, u cilindričnom fotohemijskom reaktoru „Raionnet” sa 8 simetrično postavljenih lampi, sa maksimumom emisije - 300 nm i ukupnim energetskim fluksom od 12 V/m^2 . Tečna hromatografija visokih performansi u kombinaciji sa elektrosprej jonizacionom masenom spektrometrijom je korišćena metoda za praćenje degradacije glavnih komponenti natrijum-bakar hlorofilina nakon zračenja, kao i za detektovanje mogućih novih proizvoda. Utvrđeno je da stopa fotodegradacije svih otkrivenih jedinjenja natrijum-bakar hlorofilina prati kinetiku prvog reda. Analiza je pokazala gubitak glavne komponente hlorofilina, bakar hlorina e_6 , većom brzinom u odnosu na ostale detektovane komponente komercijalnog hlorofilina, u rasponu od $7,02 \times 10^{-2} \text{ min}^{-1}$. Uočena fotoosetljivost natrijum-bakar hlorofilina je manja u poređenju sa prirodnim hlorofilima što opravdava njegovu primenu u hrani i kozmetici. Glavni detektovani fotoproizvodi su oksidovana jedinjenja hlorofilina. S druge strane, implikacija brzog gubitka bakar hlorina e_6 , može dovesti do promene potencijalnih dijetetskih koristi kao što su antimutagena i antioksidativna aktivnost.

Cljučne reči: hlorofilin, E141, degradacija, zračenje, UHPLC-DAD-ESI-MS