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Original scientific paper

Electrospray ionization mass spectrometry combined with ultra high performance liquid chromatography in the analysis of *in vitro* formation of chlorophyll complexes with copper and zinc

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Abstract: The aim of this study was to obtain a more accurate insight into the interaction of the major photosynthesis pigment, chlorophyll (Chl), with copper(II) and zinc(II) in solution using flow injection analysis combined with electrospray ionization mass spectrometry (FIA–ESI-MS), as well as combined with ultra high performance liquid chromatography with DAD detection (UHPLC-DAD). These interactions may potentially, but not necessarily, lead to the formation of Cu–Chl and Zn–Chl complexes of two different types, which has a large number, at least, disfunctional implications in the plant world. The results based on analysis of full-scan and MS/MS spectra, with and without UHPLC chromatograms, confirmed the formation of a "central type" Cu–Chl complex and a "central type" Zn–Chl complex, as well as proved the formation of a "peripheral" Zn–Chl complex, the latter one originating from a very weak coordinative interaction at the edge of the Chl structure. The employed techniques appeared to be efficient and reliable tools for studying the formation and stability of heavy metals complexes with chlorophyll, at least *in vitro*, with a considerable possibility for an assessment of real bioenvironmental behavior.

Keywords: chlorophyll; heavy metals; complexes; mass spectrometry; UHPLC.

INTRODUCTION

Plants and algae easily absorb toxic heavy metals; in the case of high metal concentrations, plants become their hyperaccumulator, such as in the case of cadmium and zinc[1] or nickel.[2] In lower concentrations, many heavy metals, such as copper and zinc, are essential micronutrients for higher plants and algae, and

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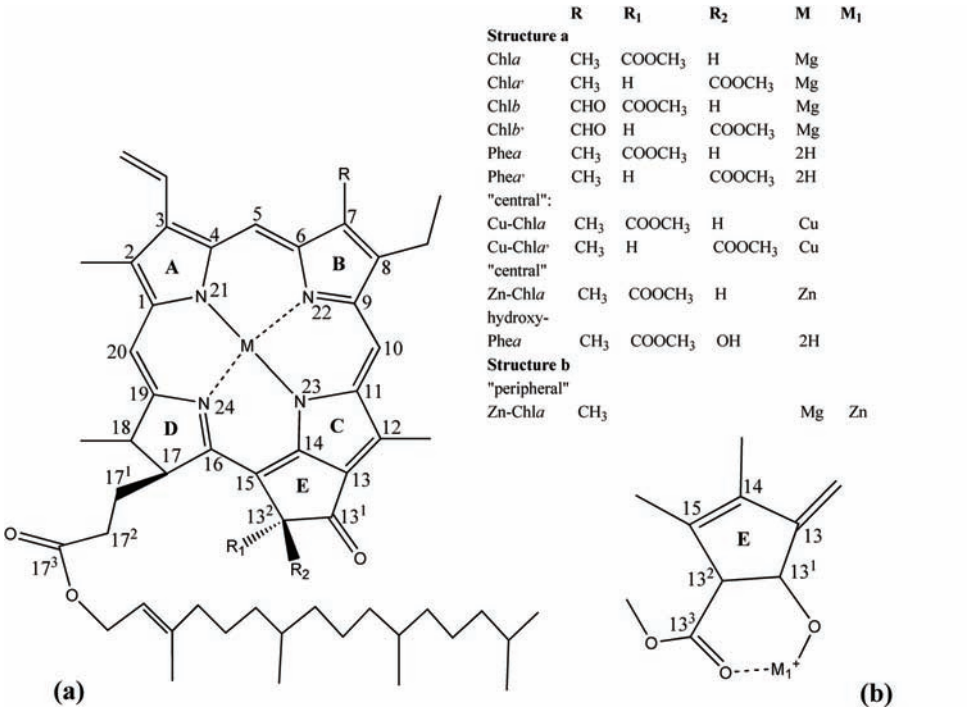
furthermore, a small Cu-protein, plastocyanin, is one of the integrative part of the electron-transport chain (ECL) of the photosynthetic apparatus of plants, connecting the photosystems I and II;[3] on the other hand zinc-porphyrins are formed during chlorophyll biosynthesis.[4] However, high external concentrations of copper and zinc may lead to many damaging effects. Zinc may be included in degradation of stromal proteins of chloroplasts.[5] Copper may affect all kinds of photosynthetic activities, such as electron transport and ATP production,[6,7] and oxygen evolution.[8] Other disrupting or toxic effects of zinc and copper on plants (and other heavy metals) were reviewed by Nagajyoti *et al.*[9] *In vivo* experiments showed that the substitution of the central magnesium (Mg) atom in chlorophyll (Chl), a major photosynthesis pigment, by heavy metals (already observed *in vitro*) may be one of the principal causes for their permanent damaging effects on the photosynthesis apparatus. Thus, the formation of chlorophyll–heavy metal complexes (Chl–HMS), even in minor proportions relative to the total Chl content, may inhibit photosynthesis completely.[10] The consequences of Chl–HMS for higher plants and green algae were discussed in details by Küpper *et al.*[10–13]

In two previous reports, the formation of copper and zinc complexes with chlorophyll *in vitro* (*i.e.*, Cu(II)–Chl and Zn(II)–Chl complexes, respectively) was proven by Vis, FTIR and fluorescence spectroscopy,[14] as well as the formation of Cu–Chl complexes in isolated photosynthetic organelles, chloroplasts, and sub-organelles, thylakoids (*ex vivo*), while the formation of the Zn–Chl counterpart was sterically prevented.[15] In a recent study, the stability of these two complexes to UV-B radiation was investigated *in vitro* and compared to those obtained with pheophytin and mesoporphyrin.[16] The higher stability of the Cu–Chl (compared to Zn–Chl) obtained confirmed the conclusions from a previous report,[14] *i.e.*, that copper forms a relatively stable "central type" of complex with Chl, with Cu(II) replacing Mg(II) in the center of the porphyrin nucleus. On the other hand, based on existing FTIR data,[14] the possible formation of a very unstable Zn–Chl chelate complex in an excess of Zn(II) ions could only be supposed, as the consequence of Zn-coordinative interactions at the edge of the Chl isocyclic cyclopentanone ring (Fig. 1), between the position of C-13^1 and C-13^3. The "central" complexes are thermodynamically favored, but the "peripheral step" may occur before their formation, and this is more enhanced in the case of Zn–Chl than in the case of Cu–Chl.[17]

Confirmation concerning the formation of Cu–Chl and Zn–Chl complexes are still rare. In the present study, the electrospray ionization mass spectrometry (ESI-MS) method was employed to provide additional proof for the formation of complexes, which should provide a deeper insight into the stabilities of the two complexes.



	R	R_1	R_2	M	M_1
Structure a					
Chl*a*	CH_3	$COOCH_3$	H	Mg	
Chl*a*′	CH_3	H	$COOCH_3$	Mg	
Chl*b*	CHO	$COOCH_3$	H	Mg	
Chl*b*′	CHO	H	$COOCH_3$	Mg	
Phe*a*	CH_3	$COOCH_3$	H	2H	
Phe*a*′	CH_3	H	$COOCH_3$	2H	
"central":					
Cu-Chl*a*	CH_3	$COOCH_3$	H	Cu	
Cu-Chl*a*′	CH_3	H	$COOCH_3$	Cu	
"central"					
Zn-Chl*a*	CH_3	$COOCH_3$	H	Zn	
hydroxy-					
Phe*a*	CH_3	$COOCH_3$	OH	2H	
Structure b					
"peripheral"					
Zn-Chl*a*	CH_3			Mg	Zn

Fig. 1. Structures of some of the main chlorophyll derivatives considered in this paper.

Recently, the employment of the ESI-MS method for a study of the interactions of chromium(III) with a series of *O*-donor humic ligands in solution was reported as informative and proved the advantages of this method for investigations of metal–ligand interactions.[18] Pursuing this goal, in the present study an attempt was made to obtain deeper insight into the formation and stability of Cu–Chl and Zn–Chl complexes using the same method.

EXPERIMENTAL

The formation of heavy metal complexes of chlorophyll, Chl–HMS, may occur under low light (“shade reaction”) and under high light conditions (“sun reaction”).[13] Since only just a minority of antenna chlorophylls (*in vivo*) is accessible to Chl–HMS formation under high light conditions,[13] the experimental procedure described below was performed under shade conditions as much as possible, inside vessels and equipment covered with aluminum foil or black cloth.[19]

Isolation of chlorophylls

Extraction of plant pigments from spinach leaves, *Spinacia oleracea* L. (from the local market), was performed using a previously published method,[20] by extraction with a mixture of methanol and petroleum ether in a 2:1 volume ratio, and a petroleum ether and diethyl ether (1:1 volume ratio) mixture used for re-extraction. The final extract was a mixture of pigments containing large amounts of various chlorophyll forms, as well as accessory pigments and carotenoids (carotenes and xanthophylls).[20] The chlorophyll fraction, a purified mixture of

various chlorophyll forms (predominantly Chl*a* and Chl*b*), was isolated from the pigment extract using open column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063–0.200 mm) and an *n*-hexane/acetone mixture as the eluent.[21] The chlorophyll fraction eluted at an eluent composition of 10:1 (*n*-hexane/acetone, v/v, respectively).[21] The total Chl content (Chl*a* + Chl*b*) in the isolated Chl-fraction was calculated as reported.[22]

Copper and zinc interaction with chlorophyll – preparation of the complexes (Cu–Chl and Zn–Chl)

Heavy metal complexes with chlorophyll, HMS–Chl (Cu–Chl and Zn–Chl), were prepared using an already published method.[16] The solvent was removed from the Chl-fraction at room temperature and the remaining solid was dissolved in ethanol/water mixture (95:5 volume ratio), to which a solution of $CuSO_4$, or $ZnSO_4$, was added. The final reaction mixture, Cu-treated or Zn-treated chlorophyll fraction, contained 5.0 mM of $CuSO_4$, or $ZnSO_4$, respectively, and 5 μM of chlorophylls (Chl*a* and Chl*b*). The reaction of the Chl molecules with Zn(II), or Cu(II) was performed by heating the reaction mixture in a reflux apparatus for 1 h at 40 °C, followed by 24 h at room temperature. The Cu-treated and Zn-treated Chl--fractions as the final solutions contained Cu–Chl and Zn–Chl complexes, as well as remaining chlorophylls and degradation products of the chlorophyll.[1,2] These solutions were used for the analyses following the hereinafter described procedures. The chlorophyll-fraction was analyzed in acetonitrile solution.

Flow injection electrospray ionization mass spectrometry analysis

Flow injection ESI-MS and MS/MS experiments were performed on an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, USA) operating in the positive ion mode by introducing the samples directly into the ion source *via* a syringe pump using the following conditions: applied flow rate, 5 μl min^{-1}; capillary voltage, 24 V; capillary temperature, 200 °C; tube lens voltage, 15 V; sheath gas flow (N_2), 18 (arbitrary units). The MS-spectra were acquired by full range covering the scale *m*/*z* 100–1000. For the fragmentation study, MS/MS experiments were performed by deploying collision-induced dissociation (CID) with the normalized collision energy of the collision-induced dissociation set at 30 and 35 eV.

Ultra high performance liquid chromatography-diode array-electrospray ionization mass spectrometry analysis

The liquid chromatography (ultra high performance chromatography – UHPLC) runs were realized using a Dionex Ultimate 3000 UHPLC+ system equipped with a diode array (DAD) detector and connected with LCQ Fleet Ion Trap Mass Spectrometer, Thermo Fisher Scientific, Germany. The separations were performed on a Hypersil gold C18 column (50×2.1 mm, 1.9 μm) from the same producer, at a 25 °C temperature. The mobile phase consisted of (A) methanol and (B) acetonitrile. A linear gradient program at a flow rate of 0.200 ml min^{-1} consisting of 0–0.75 min from 10 to 40 % (B), followed by 0.75–1.5 min to 50 % (B), 5–6 min from 50 to 10 % (B) and finishing with 10 % (B) for 4 min. An injection volume of 5 μl was used.

UV–Vis spectra were recorded on DAD-detector (with total range between 200 and 800 nm), set at two detection wavelengths, λ_{det}, 430 and 660 nm, simultaneously. The mass spectrometric analysis was performed using a LCQ 3D-ion trap mass spectrometer with electrospray ionization (ESI) operating in positive ion mode as the method of identification. The ESI-source parameters were as follows: source voltage, 4 kV; capillary voltage, 37 V; tube lens voltage, 110 V; capillary temperature, 200 °C; sheath and auxiliary gas flow (N_2), 18 and 8 (arbitrary units), respectively. The MS-spectra were acquired by full range acquisition in the

m/*z* range 100–1000. For fragmentation study, a data dependant scan was performed deploying collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 35 eV.

The cone voltage, applied to the source, can have a considerable impact on the fragmentation pattern of an ionized molecule; common ESI-MS findings of sodium (Na) or potassium (K) ion adducts with the molecules, giving shift in the corresponding peaks by 23 or 39 units, respectively, were observed together with the major fragmentation ions.[23,24] It is also common to see addition of one or more protons to the molecular ions and the corresponding Na or K adducts.[23] In some reports on fast atom bombardment (FAB) in combination with MS/MS investigations, the same effects were observed.[25]

Complexes of chlorophyll with copper and zinc were identified according to their mass spectra, and characteristic ion fragmentation within selected peaks from the corresponding UHPLC chromatogram. Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis, as well as for the simulation of some MS spectra.

All solvents used in the experiments were of HPLC or LC–MS grade. Methanol and acetonitrile used in UHPLC–MS experiments (LC–MS grade) were purchased from Baker, The Netherlands and Fisher Scientific, UK, respectively. Crystalline copper(II) sulfate pentahydrate and zinc sulfate heptahydrate were purchased from Sigma–Aldrich, Germany.

RESULTS AND DISCUSSION

The full scan mass spectra (obtained by the flow injection technique) of the investigated Chl-fraction, and the Cu- and the Zn-treated Chl-fractions are shown in Fig. 2a, b and c, respectively; the full list of the main compounds found in the chlorophyll, Cu- and Zn-treated Chl-fractions with their assignments, as well as the MS/MS spectra are given in Table I.

Chromatogram of the Cu-treated Chl-fraction, obtained from the DAD signal at a detection wavelength, $\lambda_{det.}$ = 430 nm, is shown in Fig. 3a; the corresponding Vis spectrum of the main observed peak in the chromatogram, assigned as the "central" Cu–Chl*a* complex is shown in Fig. 3b. The MS/MS spectrum of the peak belonging to the anticipated "central" Cu–Chl*a* complex, with a retention time, $t_{ret.}$ = 4.77 min, is shown in Fig. 3c; the proposed fragmentation pattern for this complex is shown in Fig. 4.

The chromatogram of the Zn-treated Chl-fraction, obtained from the DAD signal at a detection wavelength, $\lambda_{det.}$ = 430 nm, is shown in Fig. 5.

The main identified Chl-derivatives with their full chromatographic ($t_{ret.}$), Vis spectroscopic (λ_{max}) and MS (*m*/*z*) parameters found using UHPLC-DAD–MS/MS data are listed in Table II. The information from the spectral data agrees basically with the published data (given in Table II).

The Chl derivatives containing magnesium, zinc, copper could be mutually distinguished 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 metals. As copper has two natural isotopes, ^{63}Cu (69.17 %) and ^{65}Cu (30.83 %) and magnesium has three natural isotopes, ^{24}Mg (79.0 %), ^{25}Mg (10.0 %), and ^{26}Mg (11.0 %), whereas

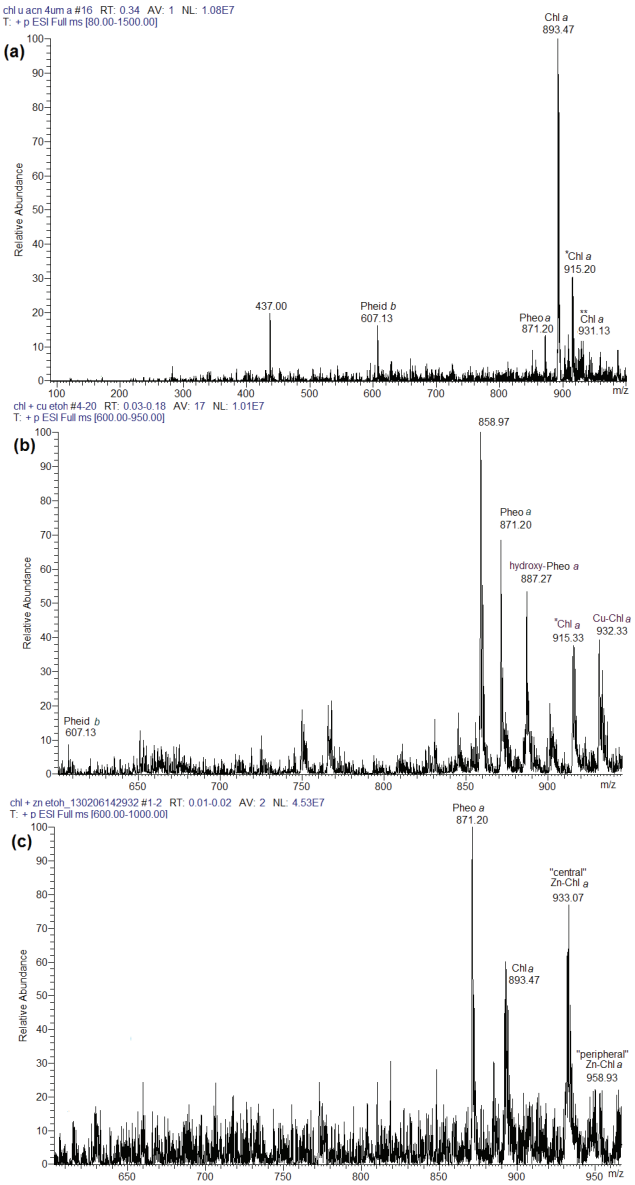


Fig. 2. Full scan MS spectra of the Chl-fraction (a), the Cu-treated Chl-fraction (b) and the Zn-treated Chl-fraction (c) obtained in the flow injection ESI-MS experiments. The main observed peaks correspond to molecular ions assigned as various Chl-derivatives. Molecular ions representing adduct-ions of Chl*a* with Na and K (at *m*/*z* 915 and 931, labeled as * and **, respectively) were observed in the full scan MS spectrum of the Chl-fraction (a); a similar situation was also observed in the Cu-treated Chl-fraction (b). A full list of the corresponding ESI-MS/MS data chosen for some of the main peaks found in the full scan MS are listed in Table I.

TABLE I. Flow injection ESI-MS/MS data of the main compounds found in the Chl-fraction, and the Cu- and Zn-treated Chl-fraction in ethanol

Compound	$[M]^+$	$[M]^+$	Main observed fragment-ions *m*/*z*	Proposed structure of the fragment-ions[b]
	Found MW/(*m*/*z*)	Average calculated MW/(*m*/*z*)[a]		
Chlorophyll *a*	893.47 (100 %)	893.50	615 $[M–278]^+$	$[M–C_{20}H_{38}]^+$
			555 $[M–338]^+$	$[M–C_{20}H_{39}CO_2CH_3]^+$
Pheophytin *a*	871.20 (100 %)	871.21	839 $[M–32]^+$	$[M–CH_3OH]^+$
			811 $[M–60]^+$	$[M–HCO_2CH_3]^+$
			593 $[M–278]^+$	$[M–C_{20}H_{38}]^+$
			533 $[M–338]^+$	$[M–C_{20}H_{39}CO_2CH_3]^+$
Pheophorbide *b*	606.39 (100 %)	606.67	574 $[M–32]^+$	$[M–CH_3OH]^+$
			548 $[MH–59]^+$	$[MH–CO_2CH_3]^+$
“Central” Cu–Chl*a*	932.33	932.74	900 $[M–32]^+$	$[M–CH_3OH]^+$
			654 $[M–278]^+$ (100 %)	$[M–C_{20}H_{38}]^+$
			593 $[M–339]^+$	$[M–C_{20}H_{39}CO_2CH_3–H]^+$
			521 $[M–411]^+$	$[M–C_{20}H_{39}CO_2CH_2CH_2––CO–HOCH_3]^+$
„Central“ Zn–Chl*a*	934.13	934.58	902 $[M–32]^+$	$[M–CH_3OH]^+$
			656 $[M–278]^+$ (100 %)	$[M–C_{20}H_{38}]^+$
„Peripheral“ Zn–Chl*a*	958.60	957.875	900 $[M–59]^+$ (100 %)	$[M–CO_2CH_3]^+$
			681 $[M–278]^+$	$[M–C_{20}H_{38}]^+$

[a]The calculated molecular masses are average values bearing in mind the isotopic abundances of each element in the proposed formula; [b]structures of the proposed fragments obtained from the literature.[25-32]

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 (M^+) of Zn–Chl derivatives was significantly broader than those of the corresponding Cu- or Mg- derivatives.[26] Software simulated spectra were compared with the experimental spectra to confirm, relativize or to reject the isotope distribution of the M^+ peaks in the full-MS experimental spectra, both flow injection and UHPLC-MS. Good agreement was found for all the investigated compounds (not shown). For example, for M^+ of “central” Cu–Chl*a* complex, a mass distribution in the *m*/*z* range 931.48 to 936.49 was found by simulation, and in flow injection MS/MS experiment, an M^+ ion at *m*/*z* 932.33 was detected.

Positive mode MS-fragmentation of the chlorophylls – a general pattern

The most abundant fragment ions in the positive mode MS/MS spectra of the different Chls usually originated from fragmentation at the C-17 and C-13 positions (Fig. 1). The former one results either in a loss of a phytyl chain (the phytadiene structure, $C_{20}H_{38}$) or in the loss of the whole $C_{20}H_{39}CO_2CH_3$ group, which

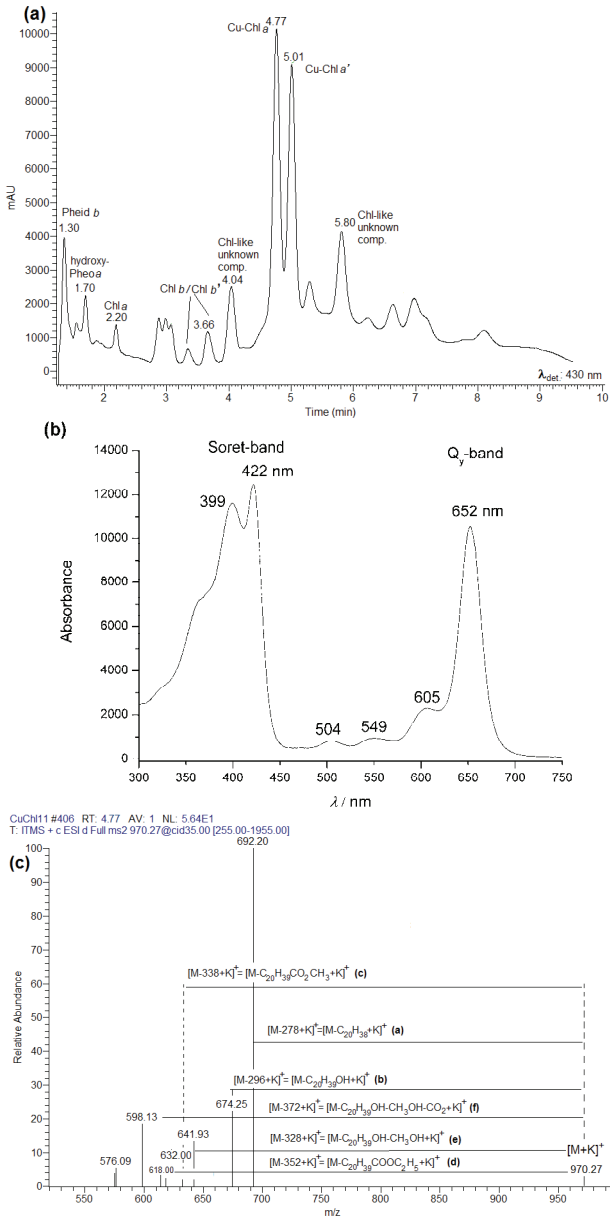


Fig. 3. Chromatogram of the Cu-treated Chl-fraction obtained from the UHPLC-DAD signal at 430 nm (a). The corresponding UV–Vis spectrum (taken from the DAD-signal (b) and the MS/MS spectrum (obtained from the UHPLC-ESI/MS measurements) by CID fragmentation of the main product, $t_{ret.}$ = 4.77 min, assigned as the "central" Cu–Chl*a* complex (c). The corresponding structure assignments are shown in Fig. 1. A possible scheme of the fragmentation pattern (linked to Fig. 3c) is shown in Fig. 4. The remaining chromatographic, UV–Vis and MS data are given in Table II.

TABLE II. UHPLC-DAD-ESI-MS/MS characterization of the main compounds found in the Chl-fraction and Cu- and Zn-treated Chl-fractions in ethanol

Assignment of the peaks	t_{ret} min	Absorbance maxima in the mobile phase[a], nm			$[M]^+$ Found MW/(*m/z*)	$[M]^+$ Average calculated[b] MW/(*m/z*)	Main observed fragment-ions in MS/MS spectra	
		I	Soret II	Q_y III			*m/z*	Proposed structure of the fragment-ions[c]
Pheophorbide *b*	1.30	–	407	665	$[MH]^+$ 607.31	606.67	575 $[MH-32]^+$ 547 $[M-59]^+$ (100%)	$[MH-CH_3OH]^+$ $[M-CO_2CH_3]^+$
Hydroxy-pheophytin *a*	1.70	–	446	650	887.20	887.21[d]	469 $[M-418]^+$	–
Chlorophyll *a* Chlorophyll *a*'	2.20 2.37	411	431	665	893.47	893.50	615 $[M-278]^+$ (100%) 555 $[M-338]^+$	$[M-C_{20}H_{38}]^+$ $[M-C_{20}H_{39}CO_2CH_3]^+$
Chlorophyll *b* Chlorophyll *b*'	3.36 3.66	–	435	654	907.37	907.50	553 $[M-354]^+$ (100%)	$[M-C_{20}H_{39}CO_2CH_3-CH_3-H]^+$
Pheophytin *a* Pheophytin *a*'	4.58 5.06	–	408	666	871.53	871.21	839 $[M-32]^+$ 811 $[M-60]^+$ 593 $[M-278]^+$ (100%) 533 $[M-338]^+$ 460 $[M-411]^+$	$[M-CH_3OH]^+$ $[M-CO_2CH_3-H]^+$ $[M-C_{20}H_{38}]^+$ $[M-C_{20}H_{39}CO_2CH_3]^+$ $[M-C_{20}H_{39}CO_2CH_2CH_2-CO-HOCH_3]^+$

TABLE II. Continued

Assignment of the peaks	$t_{ret.}$ min	Absorbance maxima in the mobile phase[a], nm			$[M]^+$ Found MW/(m/z)	$[M]^+$ Average calculated[b] MW/(m/z)	Main observed fragment-ions in MS/MS spectra	
		I	Soret II	Q_y III			m/z	Proposed structure of the fragment-ions[c]
„Central“								
Cu-Chlorophyll *a*	4.77	399	422	652	$[M+K]^+$[e]	$[M+K]^+$	692 $[M-278+K]^+$	$[M-C_{20}H_{38}+K]^+$
Cu-Chlorophyll *a*'	5.01				970.27	971.87	(100%)	
							674 $[M-296+K]^+$	$[M-C_{20}H_{39}OH+K]^+$
							642 $[M-328+K]^+$	$[M-C_{20}H_{39}OH-CH_3OH+K]^+$
						$[M]^+$	632 $[M-338+K]^+$	$[M-C_{20}H_{39}CO_2CH_3+K]^+$
						932.74	618 $[M-352+K]^+$	$[M-C_{20}H_{39}CO_2C_2H_5+K]^+$
							598 $[M-372+K]^+$	$[M-C_{20}H_{39}OH-CH_3OH-CO_2+K]^+$
“Central“[f]								
Zn-Chlorophyll *a*	2.80	410	426	658	$[M+Na]^+$	$[M+Na]^+$	677 $[M-278+Na]^+$	$[M-C_{20}H_{38}+Na]^+$
					955.25	957.57	645 $[M-310+Na]^+$	$[M-C_{20}H_{39}-CH_3O+Na]^+$
					$[M]^+$	$[M]^+$	601 $[M-354+Na]^+$	$[M-C_{20}H_{39}CO_2CH_3-CH_3-H+Na]^+$
					933.33	934.58	(100%)	
„Peripheral“								
Zn-Chlorophyll *a*	5.85	402	423	652	957.95	957.875	679 $[M-279]^+$	$[M-C_{20}H_{38}-H]^+$
							(100%)	
							603 [M-355]+	

[a]Absorbance maxima wavelengths were compared with the ones given in the literature;[29,36,38,40,41] [b]calculated molecular masses are average values keeping in mind isotopic abundances of each element in the proposed formula; [c]structures of the proposed fragments taken from the literature;[25–28,30,32] [d]literature value;[7] [e]MS/MS spectra of the molecular ion peaks at m/z 971.2 were also recorded, but are not shown in this table due to the poor fragmentation found; [f]the presence of m/z 933 and 970 peaks in the full-MS at $t_{ret.}$ = 2.8 min is typical for the “central” Zn-Chl*a* and Zn-Chl*a*–dihydrate complexes, respectively.[40] Species at m/z 955, considered as “central” Zn–Chl*a* adducts with Na, were also found. The CID-fragmentation was found only for m/z 955 species

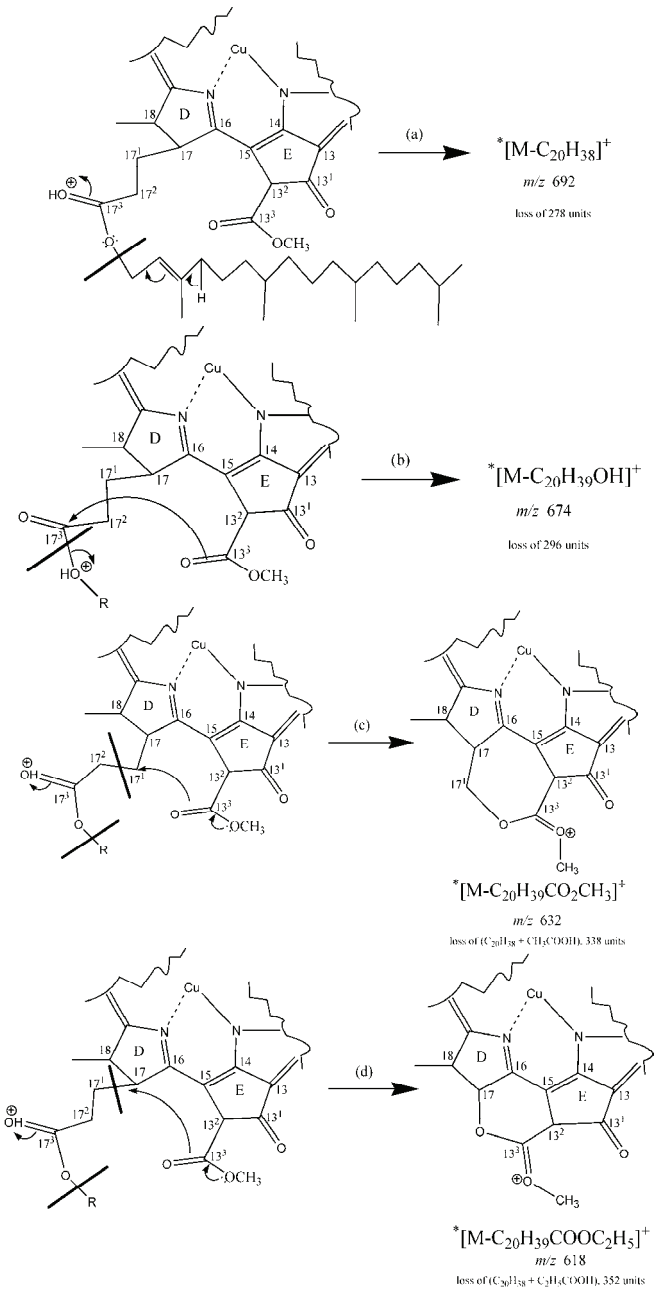


Fig 4. A proposed fragmentation pattern for the main observed compound in the Cu-treated Chl-fraction assigned as the "central" Cu–Chl*a* complex (Cu–Chl*a* and Cu–Chl*a*′), found at $t_{ret.}$ = 4.77 and 5.01 min, respectively, in the UHPLC chromatogram, based on DAD and MS/MS data. *The molecular-ion and the peaks of the fragment-ions correspond to adducts of Chl*a* with K (the corresponding masses are 39 mass units higher).

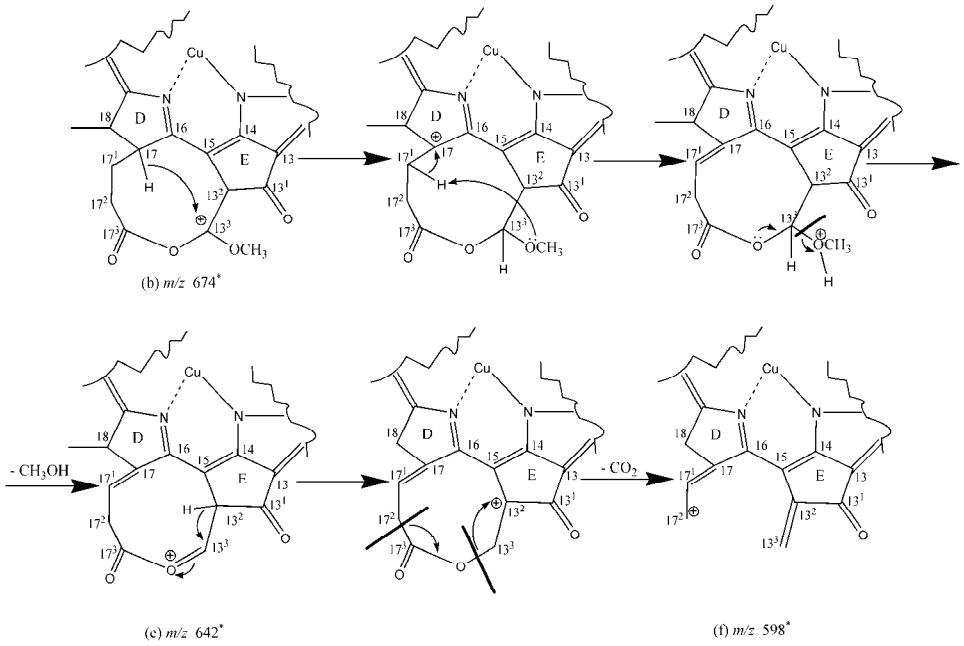


Fig 4. (Continued) A proposed fragmentation pattern for the main observed compound in the Cu-treated Chl-fraction assigned as the "central" Cu–Chl*a* complex (Cu–Chl*a* and Cu–Chl*a*′), found at $t_{ret.}$ = 4.77 and 5.01 min, respectively, in the UHPLC chromatogram, based on DAD and MS/MS data. *The molecular-ion and the peaks of the fragment-ions correspond to adducts of Chl*a* with K (the corresponding masses are 39 mass units higher).

appears in the MS/MS spectrum at *m*/*z* values corresponding to $[M–C_{20}H_{38}]^+$ = = $[M–278]^+$ and $[M–C_{20}H_{39}CO_2CH_3]^+$ = $[M–338]^+$, respectively.[27] The latter one (C-13 position) is result of a loss of CH_3OH (in the C-13^3 position) or, of the whole ester-group ($COOCH_3$ in the C-13^2 position, Fig.1), which appears in the spectrum at *m*/*z* values corresponding to $[M–CH_3OH]^+$ = $[M–32]^+$ and $[M–COOCH_3]^+$ = $[M–59]^+$, respectively.[28] The list of the obtained data presented in Tables I and II, includes, among all, the peaks resulting from a loss of 278, 338, 32 and 59 fragments.

Flow injection ESI-MS/MS spectra of Chl-, Cu- and Zn-treated Chl-fraction

Peaks at the *m*/*z* values corresponding to the molecular weights of the different Chls, such as chlorophyll *a* (Chl*α*, at 893.47) and pheophytin *a* (Pheo*a*, at 871.20), were observed in the full-MS spectra of all three samples, Chl-fraction, Cu-treated and Zn-treated Chl-fraction, as shown in Fig. 2a–c, respectively; pheophorbide *b* (Pheid*b*, at 607.13) was observed in the Chl-fraction and the Cu-treated Chl-fraction, as shown in Fig. 2a and b, respectively. On the other hand, several other peaks were also observed at *m*/*z* 858.97, 887.27, 932.33 in the Cu-treated, and at *m*/*z* 933.07, 958.93 in the Zn-treated Chl-fraction (Fig. 2b and

c, respectively). These peaks belong to hydroxy-Pheo*a* (887.27) and the "central" Cu–Chl*a* complex (932.33) in the Cu-treated Chl-fraction; the peak at *m*/*z* 858.97 (not assigned) belongs to an unknown compound (Fig. 2b). Peaks at *m*/*z* 933.07 and 958.93 found in the Zn-treated Chl-fraction (Fig. 2c) could belong to the "central" and "peripheral" chelate Zn–Chl*a* complexes, respectively. The peaks at 915 (Fig. 2a and b) and *m*/*z* 931 (Fig. 2a) are assigned as "cluster" or "adduct" ion peaks, *i.e.*, M^+ peaks of Chl*a* with metals such as Na and K, respectively.[23,24]

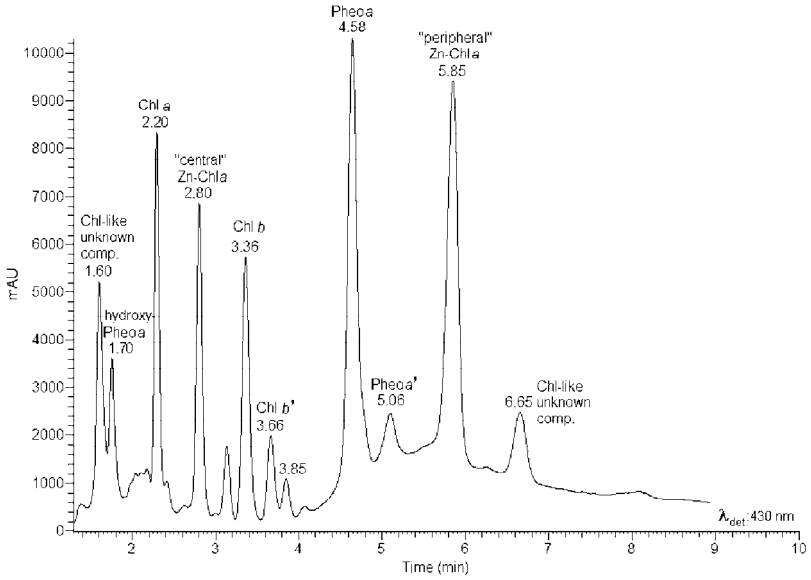


Fig. 5. Chromatogram of the Zn-treated Chl-fraction, obtained from the UHPLC-DAD signal at 430 nm. The remaining chromatographic, UV–Vis and MS data are listed in Table II.

The data taken from corresponding MS/MS spectra of the compounds found by the flow injection ESI-MS in the full scan MS spectra (given in Table I) implies fragmentation which is in agreement with the loss of groups from the C-17 and C-13 positions in the basic Chl structure shown in Fig. 1.[26–30] For example the MS/MS spectrum of *m*/*z* 893.47 (M^+) yielded fragments at 615 and 555, among the most abundant ones (Table I). The two peaks correspond to the fragmentation of the Chl*a* molecule in the C-17 position (shown in Fig. 1) with the loss of $C_{20}H_{38}$ ($[M–278]^+$) and $C_{20}H_{39}CO_2CH_3$ ($[M–338]^+$), respectively, which is in agreement with other reports.[27] On the other hand, the peak at *m*/*z* 932.33 from the Cu-treated Chl-fraction gave as the most abundant fragment in the MS/MS spectrum an ion at *m*/*z* 654 (Table I): the loss of 278 units corresponds to phytadiene, $C_{20}H_{38}$, in the C-17[3] position of the "central" Cu–Chl complex (structure shown in Fig. 1a), proving its formation in the fraction. In addi-

tion, a similar group of fragments was previously found, but in the negative mode MS/MS spectrum, originating from the molecular ion M^- at *m*/*z* 931.5, assigned as the "central" Cu–Chl*a* complex, in the extract of bright green table olives (which is called "Cu–pheophytin *a*").[29]

The peaks at *m*/*z* 933.07 and 958.93 found in Zn-treated Chl-fraction were assigned as the "central" and "peripheral" Zn–Chl*a* complexes (full-MS spectrum is given in Fig. 2c). The corresponding MS/MS fragments of the peak at *m*/*z* 934.13 (the "central" Zn–Chl*a*), 902 ($[M–32]^+ = [M–CH_3OH]^+$) and, as the most abundant, at 656 ($[M–278]^+ = [M–C_{20}H_{38}]^+$), as shown in Table I, are similar to the one found (for the Zn–Chl*a* complex) in Chl solutions subjected to FAB (fast atom bombardment)[26] as well as to APCI (atmospheric pressure chemical) ionization combined with collision activation and tandem mass spectrometry,[30] (marked as Zn–pheophytin *a* in the related papers).[26,30] On the other hand, the corresponding MS/MS fragments of the peak at *m*/*z* 958.60 (the "peripheral" Zn–Chl*a* complex) were found at *m*/*z* 900 ($[M–59]^+ = [M–CO_2CH_3]^+$), the most abundant one, and at *m*/*z* 681 ($[M–278]^+ = [M–C_{20}H_{38}]^+$) (Table I). No supporting MS-report was found to date that deals with MS and/or MS/MS fragmentation patterns that could be related to the "peripheral" heavy metal complexes of chlorophyll. However, the possibility for the formation of "peripheral" HMS complexes was already proven in other, not MS-related reports with magnesium[17] and zinc,[14] based on FTIR data.

UHPLC-DAD–ESI-MS/MS experiments

Results of chromatographic analysis (coupled with UV–Vis and MS/MS detection systems) of the investigated samples (Chl-fraction, Cu- and Zn-treated Chl-fractions) generally confirmed the results obtained by above discussed, flow injection ESI-MS/MS analysis (the chromatogram of Cu-treated Chl-fraction shown in Fig. 3a and the other data shown in Table II). The interactions of chlorophyll with zinc and copper were clearly proven.

The most intense peaks in the chromatogram of the Chl-fraction belong to Chl*a* (t_{ret} = 2.20 min), and to Chl*a*′ (Epimer*, $t_{ret.}$ = 2.37 min). The corresponding data from the MS/MS and UV-Vis spectra are presented in Table II. On the other hand, the most intense peaks in the chromatogram of the Cu-treated Chl-fraction (shown in Fig. 3a) found at $t_{ret.}$ = 4.77 and 5.01 min, were assigned to the "central" Cu–Chl*a* and Cu–Chl*a*′ complexes**. The corresponding Vis

* Epimers of chlorophylls (Chls') are almost always present in preparations of chlorophyll and its derivatives and they are naturally present in small amounts in photosynthetic organisms.[22,33] On the other hand, chlorophylls can be, in small amount, converted to the 13^2-epimers (Chls′, Fig. 1a) during the extraction processes.[33-35] Epimers of chlorophylls showed almost identical UV–Vis absorption as well as MS spectral behavior; they could be only separated by chromatography and then identified.[33]

** Epimers of Cu-chlorophyll showed identical UV–Vis absorption and MS spectra (Table II).

spectrum of the compound at $t_{ret.}$=4.77 min is shown in Fig. 3b, which is supported by the available reports.[36–38] A notable hypsochromic effect of the Q_y-band belonging to the "central" Cu–Chl*a* complex ($\lambda_{Qy\text{-}max.}$ = 652 nm, Table II) can be compared to the same band observed for Chl*a* ($\lambda_{Qy\text{-}max.}$ = 665 nm, Table II) already documented in earlier papers.[14–16,39] In addition, the MS/MS spectrum of the compound found at $t_{ret.}$= 4.77 min with mass found at *m*/*z* 970.27 ("central" Cu–Chl*a* complex, the M^+-adduct with potassium, K) is shown in Fig. 3c. The assignment of the fragments as well as a proposed pattern of MS/MS collision-induced fragmentation is shown in Fig. 4. The labels a–f in Fig. 3c correspond to the same labels of different fragmentation steps shown in Fig. 4. It is important to stress the presence of K in both molecular ions as well as in the corresponding fragments peaks in the MS/MS spectrum (Figs. 3c and 4). A major fragment at *m*/*z* 692 ($[M–278+K]^+$), corresponds to the loss of the phytyl moiety, $C_{20}H_{38}$ (Fig. 4a – fragmentation, Fig. 3c – MS/MS).[26,30] The second fragment at *m*/*z* 674 ($[M–296+K]^+$), represents phytyl chain loss at C-17[3] – in a form of phytol, $C_{20}H_{39}OH$ (Fig. 4b – fragmentation, Fig. 3c – MS/MS). The next two fragments (e, *m*/*z* 642 and f, *m*/*z* 598) are formed through a rearrangement of fragment b followed by a loss of CH_3OH and CH_3OH+CO_2, respectively (Fig. 4e and f, Fig. 3c – MS/MS spectrum). The fragment at *m*/*z* 632 ($[M–338+K]^+$) belongs to the loss of the phytyl chain, $C_{20}H_{38}$, and CH_3COOH from the C-17[1] position (Fig. 4c). The fragment at *m*/*z* 618 ($[M–352+K]^+$), corresponds to loss of $C_{20}H_{38}$ and C_2H_5COOH from the C-17 position (Fig. 4d – fragmentation, Fig. 3c – MS/MS). The fragments are also presented in Table II.

The peaks at $t_{ret.}$ = 2.20, 2.80, 3.36, 3.66, 4.58, 5.06 and 5.85 min, belonging to Chl*a*, the "central" Zn–Chl*a* complex, Chl*b*, Chl*b*′, Pheo*a*, Pheo*a*′ and the "peripheral" Zn–Chl*a* complex, respectively, are found in the chromatogram obtained from the Zn-treated Chl-fraction (Fig. 5). The three most abundant peaks at *m*/*z* 933.33, 955.25 and 970.50 dominate in the full scan MS spectrum obtained from the peak at $t_{ret.}$ = 2.80 min; they are assigned to the "central" Zn–Chl*a* complex, $[M]^+$, the $[M+Na]^+$-adduct and $[M·2H_2O]^+$ peaks, respectively. The presence of *m*/*z* 933 and 970 peaks is typical for the "central" Zn–Chl*a* and Zn–Chl*a*-dihydrate complexes, respectively (marked as Zn–pheophytin *a* and Zn–pheophytin *a* dihydrate in the paper by Nurhayati and Suendo.[40] The intensity of the *m*/*z* 970.50 peak is higher than that of the *m*/*z* 933.33 peak, probably due to more preferable existence of the "central" Zn–Chl*a*-dihydrate with coordination number 6, compared to the "central" Zn–Chl*a* complex with coordination number 4.[40] The corresponding MS/MS spectrum of the $[M+Na]^+$ adduct (*m*/*z* 955.25) is related to the peak with $t_{ret.}$ = 2.80 min, and consisted of fragments characterized by the loss of the phytyl chain from the C-17[3] position (Table II, Fig. 1a). On the other hand, the MS/MS spectrum of the anticipated "peripheral" Zn–Chl*a* complex,[14,17] with an M^+ of *m*/*z* 957.95, found at $t_{ret.}$ = 5.85 min,

consisted of two peaks, one at 679 $[M–279]^+$, the most abundant one, and at *m*/*z* 603 $[M–355]^+$ (Table II). The former one obviously originates from separation of a phytyl tail.

CONCLUSIONS

The *in vitro* formation of Cu– and Zn–Chl*a* complexes of both types (the "central" as well as the "peripheral") was confirmed by the UHPLC–ESI-MS method. This is a significant step forward because the formation of "central" Zn–Chl complexes could not be proven by Vis and FTIR spectrometry in earlier reports.[14,15,39] It should not be forgotten that the formation and stability of these complexes under *in vitro* circumstances certainly differs from those existing in *in vivo* plants and algae environments. From this point of view, the probability for the formation of "peripheral" Zn–Chl complexes seems almost negligible, taking into account the aggregated "network" of Chl molecules (both Chl*a* and Chl*b*) inside the antennas of the two photosystems, PSII and PSI.[33] Still, the reliability and high sensitivity of this method makes it an efficient tool to detect not only the formation and existence of complexes but also to study other related processes (stability, dynamics, thermodynamics, *etc*).

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И З В О Д

ЕЛЕКТРОСПРЕЈ ЈОНИЗАЦИОНА МАСЕНА СПЕКТРОМЕТРИЈА КОМБИНОВАНА СА ТЕЧНОМ ХРОМАТОГРАФИЈОМ ВИСОКИХ МОГУЋНОСТИ У АНАЛИЗИ ФОРМИРАЊА КОМПЛЕКСА ХЛОРОФИЛА СА БАКРОМ И ЦИНКОМ, *IN VITRO*

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Циљ овог рада је постизање бољег увида у интеракције хлорофила (Chl), најважнијег фотосинтетског пигмента, са бакром (II) и цинком (II), у раствору, користећи методу јонизационе масене спектрометрије комбиноване са методом течне хроматографије високих могућности са UV–Vis детекцијом (UHPLC-DAD). Ове интеракције могу потенцијално, али не и нужно, довести до формирања два различита типа комплекса хлорофила са бакром и цинком, Cu–Chl и Zn–Chl, редом, које доводе до бројних дисфункција у биљном свету. Резултати овог рада (базирани на анализи спектара добијених методом масене спектрометрије, са применом UHPLC хроматографије и без ње) потврђују формирање „централног типа" Cu–Chl комплекса, као и "централног типа" Zn–Chl комплекса, заједно са доказом о формирању и "периферног типа" Zn–Chl комплекса, који потиче од веома слабе координативне интеракције на ободу структуре молекула хлорофила. Примењен метод се показао као поуздано средство за проучавање формирања и стабилности комплекса тешких метала са хлорофилом, пре свега *in vitro*, али и са значајном могућношћу примене у реалним условима био-окружења.

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