THE IDENTIFICATION OF CHLOROPHYLL AND ITS DERIVATIVES IN THE PIGMENT MIXTURES:
HPLC-CHROMATOGRAPHY, VISIBLE AND MASS SPECTROSCOPY STUDIES

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This work represents an application of the Visible Spectrometry, High Pressure Liquid Chromatography and Electronspray Ionisation-Mass Spectrometry analysis for the identification of chlorophyll and its derivatives, pheophytin and chlorophyllide, in their purified mixtures (the chlorophyll, pheophytin and chlorophyllide fraction, respectively). The chlorophyll, pheophytin and chlorophyllide fractions were obtained by using a pigment extraction from the plant material (Spinacia Oleracea L), coupled with the column chromatography. The main components in the chlorophyll, pheophytin and chlorophyllide fractions were identified as chlorophyll a & b, pheophytin a & b and chlorophyllide a, respectively. Spectrophotometrically determined concentration ratios of the main identified pigments, chlorophyll a/b (in the chlorophyll fraction), pheophytin a/b (in the pheophytin fraction) and chlorophyllide a/b (in the chlorophyllide fraction) were: 4.95/1, 1.05/1 and 6.89/1, respectively.

Keywords: chlorophyll, pheophytin, chlorophyllide, VIS, HPLC, ESI-MS.

Introduction

Chlorophylls belong to the group of the most important bioorganic molecules; they are the principal pigments in photosynthesis, capable of light energy absorbing and its conversion to “chemical energy” by the formation of chemical compounds rich in energy (needed for the biosynthesis of carbohydrates and other compounds in photosynthetic organisms such as plants, algae and photosynthetic bacteria [1, 2]).

The chlorophylls comprise a group of more than 50 tetrapyrrolic pigments with common structural elements and function [3]. In such a way, the major photosynthesis pigment - chlorophyll (Chl) is chlorin (porphyrin derivative) in chemical terms, a cyclic tetrapyrrrole with an isocyclic cyclopentanone ring, fused at the edge of the right-bottom pyrrole ring [1-2], as shown in Figure 1. The central Mg-atom plays a coordinating role in Chl-molecule: it is

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Figure 1. Chlorophyll (A), pheophytin (B) and chlorophyllide (C) structures. C-atoms are numerated according to IUPAC nomenclature rules. In the position C-7, -R corresponding to: -CH₃ & -CHO in the cases of Chl a & Chl b, Pheo a & Pheo b, and Chlilde a & Chilide b, respectively.
bonded to N-atoms (N(21-24)) of the four pyrrole rings by two covalent and two coordinative bonds (Figure 1A). The 5-membered isocyclic ring (E) is connected to the chlorin in C(13-15) positions, while the phytol -esterified residue of propionic acid is attached to the chlorin C-17 position (Figure 1A). The groups: -CH₃ and -CHO (in a position C-7), in the case of chlorophyll a (Chl a) and chlorophyll b (Chl b), respectively (Figure 1A), make the only difference between those two chlorophylls [2, 4]. The major function of Chl a in photosynthesis is connected to light-harvesting in the antennas and light conversion processes in the Photosystems I and II (PSI and PSII), inside the photosynthetic apparatus [5-7].

In various photosynthesis "subjects" (plants, algae, bacteria), chlorophyll is often accompanied by the presence of its various derivatives, such as pheophytins (Pheo), chlorophyllides (Chlid), and many others [8-9].

Pheophytin (Pheo) is a chlorophyll derivative - demetalated chlorophyll or simply chlorophyll without central Mg-

atom [8-10], as shown in Figure 1B. The involvement of pheophytin a (Pheo a) as a primary electron acceptor in the PSII reaction center (of the photosynthetic apparatus) has been demonstrated by Klimov et al. [5], and largely strengthened by numerous biophysical measures [5]. On the other hand, pheophytin can be easily obtained during the extraction processes of the plant material, due to the liability of magnesium in the Chl-molecule [11].

Chlorophyllide (Chlid) is a derivative of chlorophyll, namely, chlorophyll without phytol-chain in C-17 position as shown in Figure 1C. It is formed in the last steps of Chl biosynthesis [5, 12], or in degradation processes like senescence of leaves [3-14]; the degradation processes include the loss of phytol (C-17) and modification of side chains of the isocyclic ring (E) of Chl (Figure 1), in the reactions catalyzed by several enzymes [13].

Table 1 gives a short preview of the natural occurrence of chlorophyll a and some of its main derivatives (Pheo a & Chlid a).

<table>
<thead>
<tr>
<th>Chlorophyll a</th>
<th>All photosynthetic algae (except prochlorophytes) and higher plants [1,2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pheophytin a</td>
<td>Photosynthetic reaction centers of higher plants, plant and algal detritus [2]</td>
</tr>
<tr>
<td>Chlorophyllide a</td>
<td>Senescent tissue, damaged centric diatoms, zooplankton faecal pellets [1,2]</td>
</tr>
</tbody>
</table>

The isolation, separation and identification of chlorophyll and its derivatives from the plants and the other photosynthetic organisms have been studied intensively during the years [1, 3, 7]. The decomposition of chlorophylls and the analysis of chlorophyll derivatives in foods have also been investigated because of their importance for the quality of fruit and vegetable products [3, 7]. The extraction and preparation (by open column chromatography) of photosynthetic pigments from different sources (in many different variations of these methods), are largely used for the isolation and separation of chlorophyll and its derivatives [13-16]. On the other hand, RP-HPLC chromatography (Reversed Phase-High Pressure Liquid Chromatography), as well as the absorbance (visible, VIS) spectrometry and MS (Mass Spectrometry) are well used methods for the analysis and identification of Chls [17-20].

The objective of this study is to represent one of the methods for fast and simple isolation, preparation and identification of chlorophyll and its derivatives (pheophytin and chlorophyllide) from the plant material (such as spinach from the local market) by using the extraction method and column chromatography as well as VIS, HPLC and E(electron) S(Spray) I(Ionization) – MS measurements.

Experimental

All experiments were performed under dim light as long as possible, and inside the vessels and equipment covered with aluminum foil or black cloth, preventing the pigment exposure to light [8].

Extraction of plant pigments. Extraction of plant pigments from spinach leaves, Spinacia oleracea L. (found in the local market), were performed by using the already published method [15]. Fresh spinach leaves were firstly depleted from the mid ribs and washed with cold water. The extraction and re-extraction mixtures were methanol and petroleum ether in a 2:1 ratio, and petroleum ether and diethyl ether (1:1), respectively. The methanol removes water from the plant material and the petroleum ether picks up the pigments before they undergo secondary reactions [21]. The diethyl ether increases the pigments solubility in the organic phase. The final extract was a mixture of pigments containing large amounts of various chlorophyll forms, as well as accessory pigments, carotenoids (carotenes and xanthophylls) [21].

Chlorophyll fraction. The chlorophyll fraction – the purified mixture of various chlorophyll forms (e.g. Chl a and Chl b) – was isolated from the pigment extract by using column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063-0.200 mm) and n-hexane/acetone mixture as the eluent [15, 18]. The n-hexane/acetone ratio was changed from the initial 1:0 to final 1:1, to allow an easier elution of the polar fractions. Chlorophyll fraction was eluted at the eluent composition 1: 0.1 (n-hexane/acetone, respectively), and then transferred in acetone.

Pheophytin fraction. The pheophytin fraction – the mixture of various pheophytin forms (e.g. Pheo a and Pheo b) – was made from the collected chlorophyll fraction by drop-wise addition of 1.0 M HCl [10]. The conversion of chlorophyll to pheophytin was completed in approximately
2 h in the dark, observed as a change of color from green to olive brown. Freshly made Pheo-stuff was then extracted by n-hexane and then dissolved in acetone.

**Extraction of chlorophyllides.** The chlorophyllides extract was obtained from dried spinach leaves (firstly depleted from the mid ribs). The chlorophyllides extraction was performed by the action of the endogenous enzyme chlorophyllase on chlorophylls, by incubating 5 g of dehydrated spinach with 100 mL acetone/0.2 M Tris–HCl buffer (pH 8.0, 1:1 v:v) for 2 h in the dark at 40 °C [3]. The final chlorophyllide extract was a mixture of pigments containing large amounts of various chlorophyllide forms, as well as their derivatives, in smaller amounts.

**Chlorophyllide fraction.** The chlorophyllide fraction – the purified mixture of chlorophyllide forms (*e.g.* Chl a and Chl b) – was isolated from the chlorophyllide extract by column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063-0.200 mm) and the n-hexane/acetone mixture as the eluent. The n-hexane/acetone ratio was changed from the initial 1:0 to final 1:10, to allow an easier elution of the polar fractions. The chlorophyllide fraction was eluted at the eluent composition 1:3:3 (n-hexane/acetone, respectively).

**VIS spectroscopy.** The spectrophotometric measurements were made on a Varian Cary-100 spectrophotometer equipped with 1.0 cm quartz cells. All spectra were recorded from 350 to 800 nm with 1.0 bandwidth. The spectra of all compounds were recorded in acetone. The contents of the investigated pigments in the chlorophyll, pheophytin and chlorophyllide fractions were determined as reported [22, 23], in the acetone. The concentrations of pigments in acetone were in the range between 10⁻⁴ and 10⁻⁵ mol/dm³.

**HPLC analysis.** The high-pressure liquid chromatography analysis of isolated chlorophyll, chlorophyllide and pheophytin fraction was performed under isocratic conditions on the apparatus: Agilent 1100 Series, Waldborn, Germany, column: Zorbax Eclipse XDB-C18, by using a iode array detector set at detection wavelengths (λₑ₀ₑ): 430 and 660 nm; the isocratic conditions were: mobile phase – acetonitrile/methanol/ethyl-acetate in the ratio 60:20:20, respectively, flow rate - 0.5 cm²/min, and temperature: 25 °C. The concentrations of the pigments were in range between 10⁻⁴ and 10⁻⁵ mol/dm³.

**ESI-MS spectrometry.** The pigment fractions were analyzed by the flow-injection ESI-MS/MS method (Electrospray ionisation- mass spectrometry), using methanol as the carrier phase. The ESI-MS/MS investigation was performed on LCQ Deca Ion Trap Mass Spectrometer (Thermo Finnigan, USA) with auxiliary equipment, by using the possibility for automatic tuning of every molecular ion in the mass spectrum to make MS/MS experiment. Mass spectra for all compounds were recorded in the most suitable mode; the value of the collision energy was increasing from 20 to 35 % until the noticeable fragmentation of smaller values of m/z, so that the initial “Parent” ion could still be seen in the spectrum. The optimization of ESI-source parameters was carried out to admit the detector light aspiration achieved by the mobilizing gas flow (10 L/min), transported under the pressure of 30 psi. The lowest voltage (50 V) was applied to ensure the efficient transport of generated ions to the analyzing module.

**Results and discussion**

**Absorption (VIS) spectra of chlorophyll, pheophytin and chlorophyllide fractions.** The absorption spectra of chlorophyll (A), chlorophyllide (B) and pheophytin (C) fraction in acetone are shown in Figure 2.

Chlorophylls as the porphyrin derivatives have two major absorption bands in the visible range, due to extended π-delocalization at the edge of cyclic tetrapyrole (porphy- rin) skeleton (Figure 1): “red” (Q-) band and “blue” (Soret or B-) band [9, 24].

The “red” and “blue” bands of Chl a (assigned as Qy- and Soret-) are located at 662 and 430 nm for acetone solutions, respectively [9-9], similar to the ones shown in Figure 2A for the chlorophyll fraction (consisting of Chl a and Chl b in the ratio 4.95/1) in acetone. The corresponding bands (Soret- and Qy-) for Chl b in acetone are located at 645.5 and 456.9 nm, respectively [8]. The small “shoulder” at 457 nm (probably due to the presence of Chl b in the mixture) is present in the absorption spectrum of the chlorophyll fraction in acetone (Figure 2A). On the other hand, the ratios of absorbance intensities for the Soret- and Qy-bands (A Soret/A Qy) are 1.23 for Chl a, and 2.82 for Chl b [8]; the corresponding A Soret/A Qy ratio for the chlorophyll fraction in acetone is 1.30 (Figure 2A). The observed small bands located in the spectral range between the Soret- and Qy- bands for the chlorophyll fraction in acetone (at 534 nm, 581 nm and 616 nm – Figure 2A) are in accordance with the corresponding literature data reported for Chl a dissolved in acetone [8].

Absorption maximums of the Soret- and Qy-bands are located at 409.5 and 665.5 nm for Pheo a in acetone [8], similar to the one shown in Figure 2B for the pheophytin fraction (consisting of Pheo a and Pheo b in ratio 1.0/1) in acetone. As compared to chlorophylls, the “red” absorption maximum of pheophytins in acetone is shifted to longer wavelengths [9]. However, the maxima in the “blue” spectral region are shifted to shorter wavelengths [8]. The ratios of the Soret- and Qy- band absorptions (A Soret/A Qy) are much higher than those to chlorophylls [22]. For Pheo a and Pheo b in acetone, A Soret/A Qy = 2.26 and 5.30, respectively [8] and for the pheophytin fraction in acetone, the corresponding A Soret/A Qy ratio is 2.33 (Figure 2B). The observed small bands located in the spectral range between the Soret- and Qy- bands for the pheophytin fraction in acetone (at 505 nm, 535 nm and 606 nm – Figure 2B) are in accordance with the corresponding literature data for Pheo a dissolved in acetone [8].
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Figure 2: The absorption spectra of chlorophyll (A), pheophytin (B) and chlorophyllide (C) fraction in acetone. The positions of the absorption maxima (Soret and Qy) are displayed within the corresponding spectra. The positions of several observed small absorption bands (between the Soret- and Qy-) are also displayed within the corresponding spectra. The concentrations of Chl and its derivatives in the corresponding fractions are in the range of \(10^{-4} - 10^{-5}\) mol/dm\(^3\). Spectrophotometrically determined concentration ratios of the main pigments in the fractions (Chl\(_a\) & Chl\(_b\) in the Chl-, Pheo\(_a\) & Pheo\(_b\) in the Pheo- and Chlidy\(_a\) & Chlidy\(_b\) in the Chlidy-) are also displayed in Figure 2 (A, B and C, respectively).

Chlorophyllide \(_a\) also has a chlorin system characterized by the absorption bands around 440 and 660 nm (in organic solvents) of almost equal intensities (\(e \approx 100.000\)) [3, 5-6, 8], similar to the one shown in Figure 2C for the chlorophyllide fraction (consisting of Chl\(_a\) and Chl\(_b\) in ratio 6.89/1) in acetone. Since the phytol tail – a main structural factor that differentiates Chl\(_a\) and Chl\(_b\) – has a very negligible absorption contribution compared to the chlorine structure, the absorption maximum positions (A\(_{Soret}\) and A\(_{Qy}\)) are almost the same – 662.0 - 664.0 nm and 431 nm in acetone, for the “red” Qy- and the “blue” Soret- bands, respectively, for both Chl\(_a\) & Chl\(_a\) [8, 22]. The corresponding bands (Soret- and Qy-) for Chl\(_b\) in acetone are located at 457.5 and 645.7 nm, respectively [8]. Small “shoulder” at 460 nm (probably due to the presence of Chl\(_b\) in the mixture) is present in the absorption spectrum of the chlorophyllide fraction in acetone (Figure 2C). The ratios of absorbance intensities for the Soret- and Qy-bands (A\(_{Soret}/A_{Qy}\)) are 1.14 for Chlidy\(_a\) in acetone [8] and 1.46 for the chlorophyllide fraction in acetone (Figure 2C). The observed small bands located in the spectral range between the Soret- and Qy- bands for the chlorophyllide fraction in acetone (at 581 nm and 614 nm – Figure 2C) are in accordance with the corresponding literature data for Chl\(_a\) dissolved in acetone [8].

HPLC-VIS analysis of chlorophyll, chlorophyllide and pheophytin fractions. This part illustrates the possibilities of applying the silica-based HPLC (with octadecyl silica, or C-18 column) coupled with absorption measurements, for the separation and identification, Chl, Pheo and Chlidy, in the corresponding mixtures, chlorophyll, pheophytin and chlorophyllide fractions, respectively.

HPLC-chromatograms of the chlorophyll, pheophytin and chlorophyllide fractions are shown in Figure 3 (A, B and C), respectively. The absorption spectra of the main compounds in the eluent mixture (mobile phase) observed in the HPLC-chromatograms at the retention times, \(t_{ret}= 13.6 \text{ & 9 min (assigned as Chl}\_a \text{ & b), 30.5 \& 19 min (assigned as Pheo}\_a \text{ & b) and 2.7 min (assigned as Chlidy}\_a\)}, were shown in the increments of Figure 3 (A, B and C), respectively. The spectra shown in increments were taken from the HPLC-VIS system (photodiode array data). The main observed peaks in this work presented HPLC-chromatograms were identified by the comparison of the absorption spectra from the photodiode array detection data (from the HPLC-VIS system) with the corresponding absorption spectra given in literature [8, 25-26]. A parallel review of the most important spectral data obtained from the corresponding absorption spectra of the observed compounds (Chl\(_a\) & b, Pheo\(_a\) & b and Chlidy\(_a\)) from the HPLC-system and the corresponding literature data [7, 27] for the same compounds in acetone (as well as in different solvents), was given in Table 2 providing the possibilities for their comparison.

High-pressure liquid chromatography (HPLC) has already been considered to be the quickest, simplest and most reproducible method for analyzing complex mixtures of pigments in food and other sources [17]. For example, the separation of chlorophylls and their derivatives by reversed-phase HPLC using an octadecyl silica (C-18) column and 100 % methanol as the eluting solvent was proposed.
by Canjura and Schwartz [28]. Leeuwe and co-workers (2006) have also proposed an optimized method for the analysis of algal pigments by using HPLC equipped with C-18 column by the gradient eluting regime [29]. Since the analysis of this type of compounds (such as chlorophylls and derivatives) predominantly uses C-18 column [30-33], the retention time always decreases in the same order (pheophytin a > chlorophyll a > pheophytin b > chlorophyll b > chlorophyllide a > chlorophyllide b), and predominantly depends on the polarity of the mobile phase [26, 30-31, 34-36]. The isocratic method that was used in this work allowed the successful separation and identification of chlorophyll and its derivatives, pheophytin and chlorophyllide.

Table 2. The parallel review of the most important spectral data obtained from the corresponding absorption spectra of the observed compounds (Chl a & b, Pheo a & b and Child a) - from the HPLC-VIS system (left) and the corresponding literature data reported for the same compounds (right).

<table>
<thead>
<tr>
<th>Compounds in acetone</th>
<th>Experimental data</th>
<th>Literature data [7]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compounds in HPLC-mobile phase</td>
<td>Absorption maxima positions/nm</td>
<td>Band ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A&lt;sub&gt;529&lt;/sub&gt;/A&lt;sub&gt;665&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A&lt;sub&gt;536&lt;/sub&gt;/A&lt;sub&gt;665&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A&lt;sub&gt;700&lt;/sub&gt;/A&lt;sub&gt;665&lt;/sub&gt;</td>
</tr>
<tr>
<td>Chl a</td>
<td>13.6</td>
<td>431.0</td>
</tr>
<tr>
<td>Chl b</td>
<td>9.0</td>
<td>461.0</td>
</tr>
<tr>
<td>Pheo a</td>
<td>30.5</td>
<td>409.0</td>
</tr>
<tr>
<td>Pheo b</td>
<td>19.0</td>
<td>434.5</td>
</tr>
<tr>
<td>Child a</td>
<td>2.7</td>
<td>431.0</td>
</tr>
<tr>
<td>Child b</td>
<td>not found</td>
<td>-</td>
</tr>
</tbody>
</table>

*Data are given in the value range for the compounds dissolved in different solvents, e.g. methanol, acetone, diethyl-ether, HPLC-mobile phase given in the literature.

HPLC-chromatogram of the chlorophyll fraction shows four good resolved peaks at retention times, 9.0, 9.8, 13.6 and 16.0 min (Figure 3A). Two major peaks at t<sub>ret</sub> 9.0 and 13.6 min correspond to Chl b and Chl a, respectively. The replacement of CH<sub>3</sub> group (in Chl a) with -CHO group (in Chl b) at the position C-7 (Figure 1A) increases the polarity of the b chlorophylls [26]; since more polar than Chl a, Chl b appears on the shorter retention time, bearing in mind the predominantly non-polar C-18 type of the column [26]. The absorption spectra of the corresponding compounds in the mobile phase (taken from the peaks at the t<sub>ret</sub> = 9.0 and 13.6 min by using HPLC-VIS – photodiode ray measurements – increment of Figure 3A) showed good agreement with the literature data for the Chl b and Chl a, respectively [30-31, 34-35] as well as with the literature data given in Table 2. The other two observed components at retention times 9.8 and 16 min are C-13<sup>2</sup> epimers of Chl b and a – Chl b′ and a′, respectively<sup>1</sup>. Chl-epimers have identical absorption spectra with the “regular” Chls (spectra not shown), but different chromatographic abilities [2]. Chl a eluted before Chl a′ (Figure 3A) because the –CHOOC<sub>2</sub>H<sub>3</sub> substituent at the C-13<sup>2</sup> position in the Chl a molecule (Figure 1A) is not on the same plane of the C-17<sup>2</sup> phytll group and is therefore less hindered and thus more polar than Chl a′ [26]. Similarly, Chl b eluted before Chl b′ (Figure 3A). Epimers of chlorophylls (Chls) are almost always present in the chlorophyll and its derivatives preparations: they are naturally present in small amounts in photosynthetic organisms [2, 22]; on the other hand, chlorophylls can be, in small amount, converted to the 13<sup>2</sup>-epimers (Chls′) during the extraction processes<sup>2</sup> [2, 37].

Similarly to the observed HPLC-chromatogram of the chlorophyll fraction (Figure 3A), HPLC- chromatogram of the pheophytin fraction also consisted of four good resolved peaks (but at longer retention times than the ones observed for Chl-fraction) at 19.0, 21.7, 30.5 and 34.0 min, assigned as Pheo b, Pheo b′, Pheo a and Pheo a′, respectively (Figure 3B). Since pheophytins (the demetalated chlorophylls) are less polar than the corresponding chlorophylls, their longer t<sub>ret</sub> is expected in non-polar C-18 type of the column, as shown in Figure 3(A,B). Two major peaks at t<sub>ret</sub> 19.0 and 30.5 min correspond to Pheo b and Pheo a, respectively. The absorption spectra of the corresponding compounds in the mobile phase (taken from the peaks at the t<sub>ret</sub> = 19.0 and 30.5 min by using HPLC-VIS - photodiode ray measurements – increment of Figure 3B) showed good agreement with the literature data for the Pheo b and Pheo a, respectively [30-31, 34-35] as well as with the literature data given in Table 2. The other two observed components at retention times 21.7 min and 34.0 min (Figure 3B) are C-13<sup>2</sup> epimers of Pheo a and a – Phe b′ and a′, respectively; Pheo a′ and b′ usually accompany the corresponding Pheo a and b in chlorophyll and its derivatives preparations [2, 22, 37].

HPLC-chromatogram of the chlorophyllide fraction shows two good resolved peaks at retention times 2.7 min and 3.0 min (Figure 3C). The first peak (at 2.7 min) is Child a and the second (at 3.0) could be Child a′ – the absorption spectra of both compounds in the mobile phase (taken from the HPLC-VIS system) are the same, which is characteristic of epimers [2].

<sup>1</sup>Chl a′ is 13<sup>2</sup>(S)-epimer of Chl a, while, Chl a is the 13<sup>2</sup>(R)-epimer; the same thing is with other epimers of Chl and its derivatives, e.g. Chl b′ & Chl b, Pheo a′ & Pheo a, Pheo b′ & Pheo b and etc.

<sup>2</sup>Chl′ (13<sup>2</sup>(S)-epimer) is less stable than Chl itself [2].
Figure 3. HPLC profile of chlorophyll (A), pheophytin (B) and chlorophyllide (C) fractions. The pigment contents expressed as concentration ratios of the main compounds in the fractions, \( C_{\text{Chl}a} / C_{\text{Chl}b} \) (in the chlorophyll fraction), \( C_{\text{Pheo}a} / C_{\text{Pheo}b} \) (in the pheophytin fraction) and \( C_{\text{Chl}id}a / C_{\text{Chl}id}b \) (in the chlorophyllide fraction) were: 4.95/1, 1.05/1 and 6.89/1, respectively. The detection was carried out at 660 nm. Chromatographic conditions are described in the Experimental part. The corresponding absorption spectra of the Chl \( a \) & \( b \), Pheo \( a \) & \( b \) and Chl \( a \) in the mobile phase, taken from the peaks at the retention times 9.0 & 13.6, 19.0 & 30.5 and 2.7 min (from the photodiode array data), were displayed in the increments of Figure 3(A, B and C), respectively.

The chlorophyllides with an unesterified propionic acid group at C-17 - Figure 1C are much more polar than the corresponding chlorophylls: they were the first eluted from the non-polar C-18 column (Figure 3). Chlorophyllide \( a \) (peak at 2.7 min) is dominant in the chlorophyllide fraction, as shown in Figure 3C. Spectral data (from the absorption spectrum of the compound in the mobile phase at the \( t_{\text{ret}} = 2.7 \) min) have shown good agreement with the spectral data for Chl \( a \) given in literature, as shown in the Table 2. On the other hand, a detectable peak (which could be assigned to Chl \( b \)) at \( t_{\text{ret}} < 2.7 \) min is not found in HPLC-chromatogram of the chlorophyllide fraction (Figure 3C): probably due to Chl \( a \) small amounts determined spectrophotometrically – almost 7 times lower Chl \( b \) than Chl \( a \) concentration, and/or simply, Chl \( b \) high polarity has as a consequence its getting around the non-polar (C-18) column together with the solvent used for the sample (acetone).

MS spectra of chlorophyll, pheophytin and chlorophyllide fractions. Electrospray-ionization mass spectrometry, ESI-MS is used for the detection and characterization of one or more ionic species in the solution, and is confirmed as very useful in studies of various systems [38-40]. ESI-MS allows the acquisition of mass spectra directly from a liquid sample [38, 41]. Depending on the charge of ions in the solution, positive or negative ion mode can be applied (ESI$^+$ or ESI$^-$, respectively) in order to obtain optimum results [39]. If there is the ambiguity in the interpretation of spectra, a high resolution mass spectrometry and MS/MS analysis leads to almost unequivocal identification [39]. On the other hand, the composition of the solutions subjected to the analysis is conditioned by significant limitations: not to apply high ionic strength because even relatively low concentrations of nonvolatile components (e.g. Na$^+$ ions) can be disturbing [40].

In the MS spectra of the chlorophyll, pheophytin and chlorophyllide fractions (not shown), molecular peaks at the \( m/z \) values which correspond to the molecular weights of the found compounds from the HPLC-VIS results were observed: Chl \( a \) (893.8), Pheo \( a \) (871.7), Chl \( a \) (614.2) among the Chl \( a \), Pheo \( b \) and Chl \( b \). Molecular ion radicals, [M]$^+$, for all pigment mixtures used in this work (Chl-, Pheo-, and Chl- fractions) were observed in the positive ion mass spectra. The MS/MS spectra of Chl \( a \), Pheo \( a \) and Chl \( a \) from the chlorophyll, pheophytin and chlorophyllide fractions are shown in Figure 4 (A, B and C), respectively.

The most abundant fragment ions in positive ion mass spectra of chlorophyll and its derivatives usually correspond to the fragmentation with the loss of groups from the C-17 and C-13$^+$ positions (Figure 1) [26, 30, 39, 42-43]. The former ones can be the result of the loss of the phytadiene (as the phytadiene, C$_{20}$H$_{36}$) or Ch$_{4}$COOC$_{20}$H$_{38}$ group – (C-17 position in Figure 1) – which appeared in the mass spectrum at \( m/z \) values corresponding to [M-C$_{20}$H$_{36}$]$^+$ = [M-278]$^+$ and [M-CH$_{4}$COOC$_{20}$H$_{38}$]$^+$ = [M-338]$^+$, respectively [26]. The last ones can be the result of the loss of CH$_{3}$OH (in C-13$^+$ position) or the whole ester-group (COOCH$_{3}$ in C-13$^+$ position) – (Figure 1) – which appeared in the mass spectrum at \( m/z \) values corresponding to [M-CH$_{3}$OH]$^+$ =
[M-32]+ and [M- COOCH$_3$]+ = [M-59]+, respectively [18].

In the mass spectrum of the chlorophyll fraction, the observed peak at $m/z$ 893.8 was assigned as a molecular ion-peak of Chl $a$ (not shown). The second fragmentation of the same peak found at $m/z$ 893.8 gave MS/MS spectrum typical for the Chl $a$, as shown in Figure 4A. In the corresponding mass spectrum of Pheo $a$ two most abundant fragmentation-ions were clearly observed: at $m/z$ 533.5 (corresponding to [M-CH$_3$COOC$_{20}$H$_{39}$]+ = [M-338]$^+$) and 593.3 (corresponding to [M-C$_{20}$H$_{38}$]+ = [M-278]$^+$) among the most abundant molecular ion-peak found at 871.7 (Figure 4B) which is in agreement with the literature data [26].

In the mass spectrum of the chlorophyllide fraction, the observed peak at $m/z$ 614.2 was assigned as the molecular ion- peak of Chl $a$ (not shown). The second fragmentation of the same peak found at $m/z$ 614.2 gave MS/MS spectrum typical for the Chl $a$, as shown in Figure 4C. Fragment ions corresponding to [M - CH$_3$OH]$^+$ and [M - COOCH$_3$]+, at 583.1 and 555.2 $m/z$ respectively were found in the mass spectrum of the Chl $a$ (Figure 4C) and they are in agreement with the literature data [38-39, 43].

**Conclusion**

This work represents an application of VIS spectrometry, HPLC-VIS and ESI-MS/MS analysis used for the identification of chlorophyll and its derivatives pheophytin and chlorophyllide in their purified mixtures (the chlorophyll, pheophytin and chlorophyllide fraction, respectively). As expected, the main components in the chlorophyll, pheophytin and chlorophyllide fractions were identified as chlorophyll $a$ & $b$, pheophytin $a$ & $b$ and chlorophyllide $a$, respectively.

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**References**

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Ova studija se bavi primenom VIS-spektrometrije, HPLC-hromatografije i Elektron-sprej jonizacione masene spektrometrije, u analizi i identifikaciji hlorofila i nekih njegovih derivata kao što su feofitin i hlorofilid, u njihovim pročišćenim smešama (hlorofilna, feofitinska i hlorofilidna frakcija, respektivno). Hlorofilna, feofitinska i hlorofilidna frakcija su dobijene iz pigmenatnih ekstrakata iz biljnog materijala (Spinacia Oleracea L), njihovim prečišćavanjem na koloni. Najvažnije komponente hlorofilne, feofitinske i hlorofilidne frakcije su identifikovane kao hlorofil $a$ & $b$, feofitin $a$ & $b$ i hlorofilid $a$, respektivno. Odnos koncentracija datih pigmenta (hlorofil $a/b$, feofitin $a/b$, hlorofilid $a/b$) je određen spektrotometrijski i iznosi, 4.95/1 za hlorofilnu, 1.05/1 za feofitinsku, i 6.89/1, za hlorofilidnu frakciju.

Izvor