HEMATOPORPHYRIN DERIVATIVES: THE ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY - DIODE ARRAY - ELECTROSPRAY IONIZATION - MASS SPECTROMETRY ANALYSIS

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(ORIGINAL SCIENTIFIC PAPER)
UDC 612.461.27:543.51:543.544

Recent advances in the column technology have led to the development of small particles as stationary phase carriers in reversed-phase liquid chromatography for separation of large scale of molecules. This work represents an application of UHPLC (ultrahigh performance liquid chromatography with 1.9 μm C-18 particles) coupled with ESI-MS (electro spray ionization-mass spectrometry) and DAD (diode array) analysis for the identification of the mixture of porphyrins such as hematoporphyrin and protoporphyrin derivatives, widely known photosensitizers used in medicine. Five porphyrin derivatives were separated and identified in the hematoporphyrin mixture: hematoporphyrin IX, protoporphyrin IX, two hydroxyethyl-vinyl-derivatives and methyl-ester of protoporphyrin IX.

Keywords: hematoporphyrin IX; protoporphyrin IX; liquid chromatography; mass spectrometry.

Introduction

Porphyrins are a large class of natural and synthetic pigments consisting of four pyrrole rings linked by four methine bridges giving one tetrapyrrole aromatic ring [1]. A general porphyrin structure with IUPAC-numbering is shown in Figure 1A. The main physiological signature lies in the heme and chlorophyll pathways, although porphyrins are widely distributed in the nature [2]. On the other hand, porphyrins have many uses: in medicine, in photodynamic therapy (PDT) of several tumor types [3-5], or in the conversion of solar to electric energy in a new type of photoelectrochemical biofuel cells [6].

Hematoporphyrin is a widely used porphyrin-photosensitizer in the photodynamic therapy of tumors, which is also used as a precursor in the oligomerization produced new PDT-sensitizers [7]. Hematoporphyrin derivatives belong to the family of porphyrin compounds found in the products used in PDT such as Photofrin® and Photodit® [7,8]. On the other hand, high performance liquid chromatography (HPLC) and mass spectrometry (MS) are indispensable experimental techniques for the separation, identification and quantitation of porphyrins such as hematoporphyrin [1]. Advances in the HPLC column technology and instrumentation lead to the use of ultrahigh performance liquid chromatography (UHPLC) coupled with mass spectrometry for improving the quality of the porphyrins research [9]. This work represents the application of ultrahigh performance liquid chromatography coupled by diode array and mass spectrometry detection (tandem mass spectrometry - MS/MS) for separation and identification of the hematoporphyrin derivatives mixture. The structures of hematoporphyrin IX (HP – 7,12-Bis(1-hydroxyethyl)-3,8,13,17-tetramethylporphyrin-2,18-dipropionic acid), protoporphyrin IX (PP – 3,8,13,17-tetramethyl-7,12-divinylporphyrin-2,18-dipropionic acid) and the related hydroxyethyl-vinyl-derivatives discussed in the paper are shown in **Figure 1.**

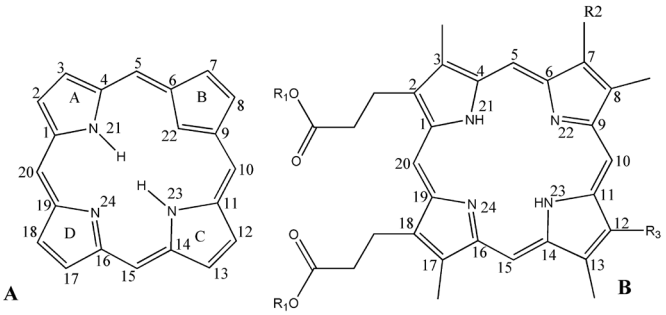

Figure 1. Porphyrin skeleton structure with IUPAC-numbering (A); Structure of hematoporphyrin derivatives (B). Hematoporphyrin IX (R_1=H; R_2=R_3= -CH(OH)CH_3); Protoporphyrin IX (R_1=H; R_2=R_3=-CH=CH_2); methyl ester of protoporphyrin IX (R_1= -CH_3; R_2=R_3= -CH=CH_2); 7(12)-(1-hydroxyethyl)-12(7)-vinyl isomers of 3,8,13,17-tetramethyl-2,18-dipropionic acid (R_1=H; I-R_2= -CH(OH)CH_3 & I-R_3= -CH=CH_2; II-R_2= -CH=CH_2 & II-R_3=-CH(OH)CH_3).

Material and methods

The hematoporphyrin mixture was a gift from the Hugo Scheer Laboratory, Botanisches Institute, LMU University Munich, Germany. Methanol and water were purchased from Baker, The Netherlands (LC–MS grade, used in UHPLC–MS experiments).

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The manuscript received: May, 29, 2017.
Paper accepted: June, 30, 2017.

Sample preparation

The sample was prepared by dissolving hematoporphyrin in methanol to give the concentration of $1.5 \cdot 10^{-2}$ g dm^{-3}.

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

Liquid chromatography (ultra-high performance chromatography – UHPLC) runs were carried out by using a Dionex Ultimate 3000 UHPLC+ system equipped with a diode array (DAD) detector and also connected with LCQ Fleet Ion Trap Mass Spectrometer, Thermo Fisher Scientific, the USA. The separations were performed on Hypersil gold C18 column (50×2.1 mm, 1.9 µm) of the same producer. The mobile phase consisted of 0.1% formic acid in (A) water and (B) methanol. A gradient program at the flow rate of 0.200 ml min^{-1} was used: the isocratic run during 3.5 min with 80% (B) was followed by the gradient to 5.0 min from 80 to 99% (B) and to 7.0 min at 99% (B), and at 7.1 min back to 80% (B) followed by isocratic 80% (B) to 15th min. The injection volume was 3 µl.

Absorption UV-Vis spectra were recorded on DAD-detector (with the total range between 300 and 750 nm), set at three detection wavelengths, λ_{det}, 395, 400 and 370 nm, simultaneously. The mass spectrometric analysis was performed using a LCQ 3D-ion trap mass spectrometer with electrospray ionization (ESI) in a positive ion mode. The ESI–source parameters were as follows: source voltage 4.5 kV, capillary voltage 48 V, tube lens voltage 115 V, capillary temperature 300 ºC, nitrogen sheath and auxiliary gas flow 32 and 8 arbitrary units, respectively. MS-spectra were obtained by the full range acquisition of m/z 100–1000. For the fragmentation study (MS/MS), a data dependent scan was performed by deploying the collision–induced dissociation (CID) with the normalized collision energy set at 20 eV. Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis.

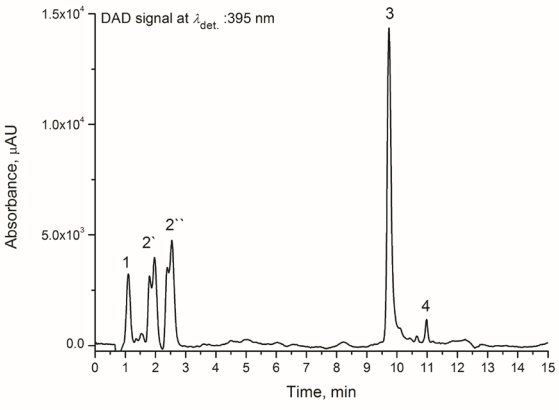

Figure 2. UHPLC-DAD chromatogram of the thematoporphyrin derivatives mixture at $\lambda_{det.}$ 395 nm. Hematoporphyrin IX (peak No.1), two vinyl-hydroxyethyl isomers (peaks No. 2' and 2"), protoporphyrin IX (peak No.3) and methyl-ester of protoporphyrin IX (No.4).

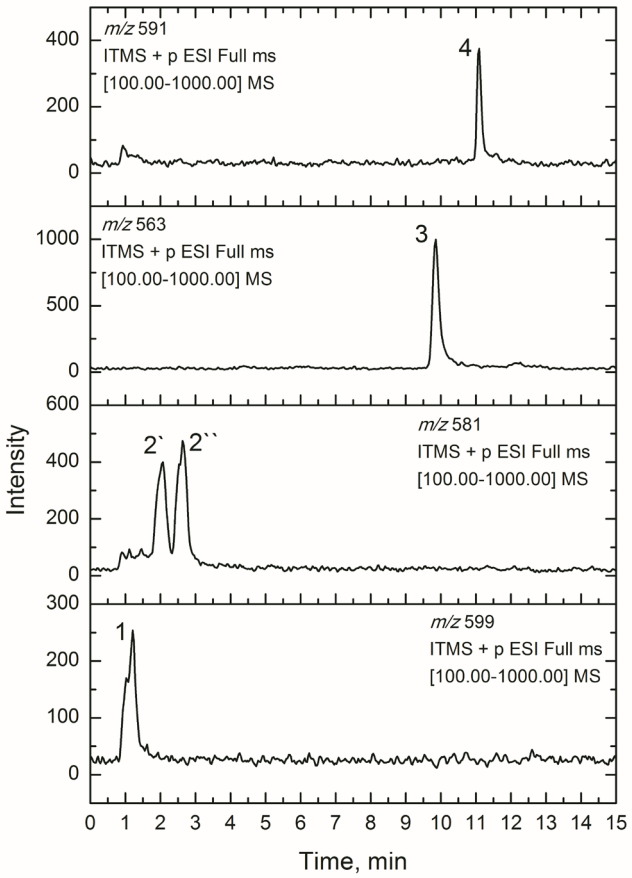

Figure 3. UHPLC-ESI MS profile of the hematoporphyrin derivatives mixture. Various detected ions are indicated, in the order of their elution time: ions *m/z* 599, 581, 563 and 591; the corresponding peaks are labeled with No.1, 2, (two isomers 2' and 2"), 3 and 4, respectively.

Results and discussion

Recent advances in the HPLC column technology have led to the development of smaller particles as stationary phase carriers in the reversed-phase liquid chromatography. For the separation and characterization of porphyrin derivatives mixture, the powerful UHPLC-DAD-ESI MS/MS method was used in this work. Chromatograms obtained from the DAD-signal at 395 nm, as well as from the corresponding MS molecular ion peaks values, are shown in Figures 2 and 3, respectively. The absorption spectra are shown in Figure 4 for most of the identified compounds. The corresponding MS/MS spectra of detected molecular ions obtained by helium collision at 20 eV are shown in Figure 5.

Five porphyrin derivatives were detected in the mixture: hematoporphyrin IX (peak No.1), two vinyl-hydroxyethyl isomers (peaks No. 2' and 2"), protoporphyrin IX (peak No.3) and methyl-ester of protoporphyrin IX (No.4), as shown in Figures 2 and 3, in the corresponding chromatograms. The results are in accordance with the principle of the reversed phase liquid chromatography used in this work, and also

confirm the structure of porphyrin derivatives established by mass spectrometry. The corresponding m/z values for molecular ions peaks, $[M+H]^+$, are in accordance with the proposed identification of the analyzed hematoporphyrin mixture. The most polar hematoporphyrin IX (Figure 1, $R_2 = R_3 = –CH(CH_3)OH$) was eluted at the beginning of the separation process (peak No.1, $t_{ret.}$ = 1.1 min, $[M+H]^+$: *m/z* 599). The retention time of methyl-ester of protoporphyrin IX (No.4) is the longest, $t_{ret.}$ = 11.4 min with the molecular ion peak value $[M+H]^+$ at *m/z* 591. Two isomers with detected $[M+H]^+$ at the same m/z value (581) belong to vinyl-hydroxyethyl isomers, peaks No.2' and 2" at $t_{ret.}$ = 1.97 and 2.54 min. Protoporphyrin IX (peak No.3, $[M+H]^+$: *m/z* 563) was eluted at tret.= 9.7 min.

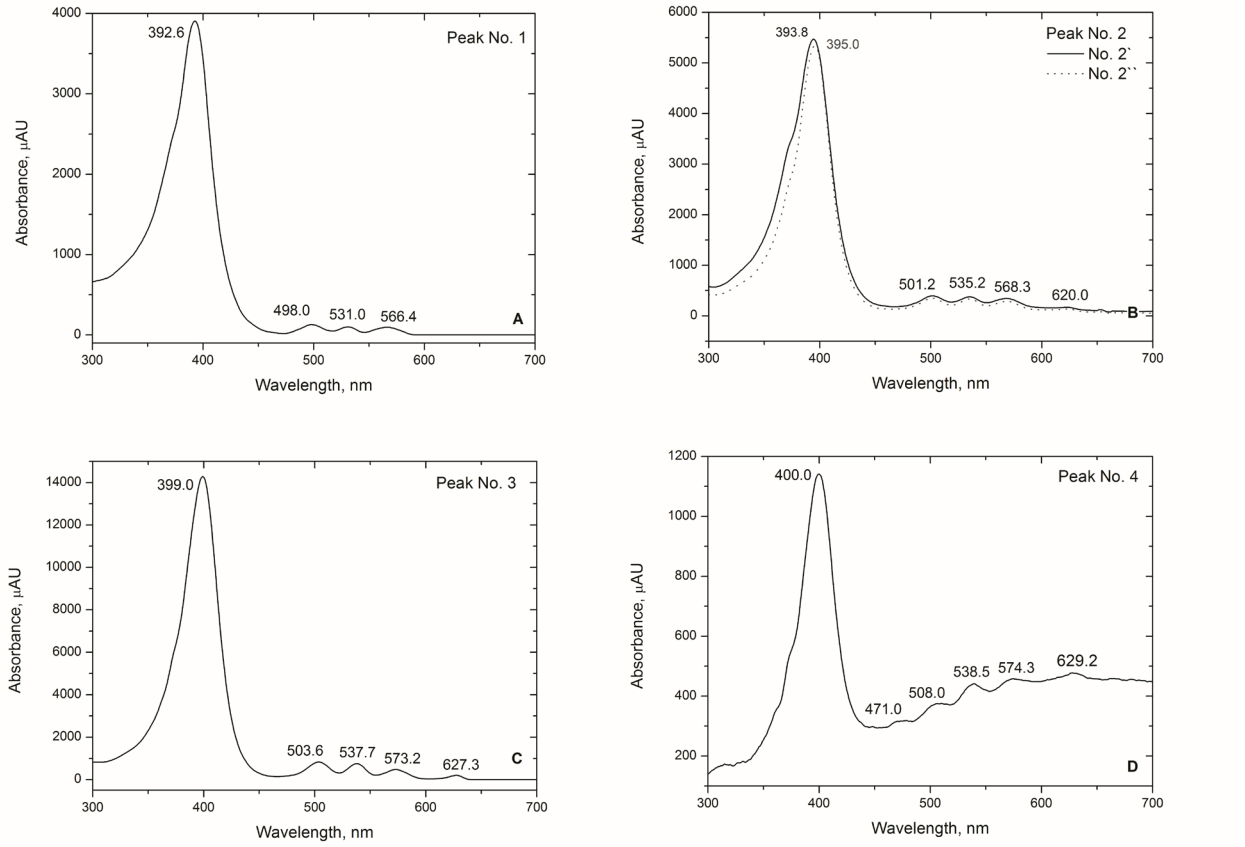

Figure 4. Absorption spectra of selected porphyrins: hematoporphyrin IX (A), two vinyl-hydroxyethyl isomers (B), protoporphyrin IX (C) and methyl-ester of protoporphyrin IX (D).

Absorption spectra of porphyrins have two major absorption bands in the visible range, due to extended π-delocalization at the edge of the cyclic tetrapyrrole (porphyrin) skeleton (Figure 1): a "blue" (Soret or B) band in the range of 380-500 nm and "red" (Q) bands in the range of 500-750 nm [10-13]. The Soret-band involves the transition from the ground state to the second excited state ($S0 \rightarrow S_2$) and the range of absorption depending on whether the porphyrin is *β*-pyrrole (positions 2, 3, 7, 8, 12, 13, 17 and 18) or meso (4, 10, 15 and 20) -substituted (Figure 1). The substitution with a weak transition to the first excited state ($S0 \rightarrow S_1$) in the range between 500-750 nm (the Q bands-I, II, III, IV) is due to the conjugation of 18 π - electrons in the porphyrin macrocycle (Figure 1). Peripheral substituents variations on the porphyrin ring often cause minor changes to the intensity and wavelength [13]. The structures of separated porphyrins (as shown in this work) consisted of *β*-substitution: four methyl groups (positions C-3,8,13,17) and propionic acid chains (C-2,18 – Figure 1B). The structure differences between detected porphyrins in C-7,12 positions caused minor changes in the corresponding absorption spectra, as expected. However, the slight "red" shift was observed for PP, 7,12-divnyl derivative (Figure 4C) in comparison to HP (1-hydroxyethyl derivative, Figure 4A) in the whole absorption range due to extended π-delocalization by vinyl-groups in *β*-positions (Figure 1B), proving the identified structures.

Mass spectra of the porphyrins usually consisted of the molecular ion peak at m/z value of $[M+H]^+$ and fragmentation ions produced by the loss of peripheral groups with heteroatoms or double bonds such as COOH and $-CH=CH_2$, respectively, without porphyrin macrocycle cleavage due to the not-interrupted π-electrons conjugation of tetrapyrrole macrocycle [1,14]. In the MS/MS spectrum of HP IX ($[M+H]^+$ - *m/z* 599), only one fragment ion peak at *m/z* 511 was found which can be obtained by the loss of two hydroxyethyl groups as $CH(OH)=CH_2$ or two CO_2 ($[MH-88]^+$) from the positions 7 and 12 or 2 and 18, respectively (Figure 1) - [7].

Two isomers 7-(1-hydroxyethyl)-12-vinyl- and 7-vinyl-12-(1-hydroxyethyl)- derivatives of 3,8,13,17-tetramethylporphyrin-2,18-dipropionic acid were separated in the retention scale between 1.5 and 2.5 min, with similar MS/MS spectra consisting of molecular ion at m/z 581 (Figure 5A,B). Mass spectrum of the first isomer (peak No.2'at 1.97 min) consisted of fragment-ion peaks detected at m/z 564, 537 and 479 (100%) corresponding to the loss of 17 (OH), 44 (CO_2 or CH_2=CHOH) and 102 units, respectively (Figure 5A). The presence of -CH_3 substituent in the molecule (from one of the positions 3,8,13 or 17) can be proved by the loss of methyl-radical from the fragment ion which is produced by the loss of two *m/z* 44 units (2 x CO_2), followed by protonation [1]. So, the last fragment ion of the isomer No.2' detected at m/z 479 (loss of 102 units) can be explained in a similar manner: the loss of two CO_2 followed by the loss of CH_3 and later protonation (581-2x44-15+1=479). The mass spectrum of the second isomer (peak No.2" at 2.54 min) consisted of fragment ion peaks detected at *m/z* 537, 522 (100%) and 434, corresponding to the loss of 44 (CO_2 or CH_2=CHOH), 59 (CH_2COOH) and 147 (2x $CH2CH_2COOH$) units, respectively (Figure 5B). A precursor ion at *m/z* 563 (comp.No.3, Figures 1,2) is fragmented by the loss of one or two CH_2COOH radicals to give product-ions at m/z 504 (the loss of 59 units from the C-2 or C-18 position), as well as *m/z* 445 (the loss of 2 x 59 units from the C-2 & C-18 positions), respectively [1], as shown in the corresponding MS/MS spectrum in Figure 5C. The elimination of H_2O and two CO_2 from a protonated molecule was also observed, giving an ion at m/z 545 (loss of 18 units) and an ion at m/z 475 (loss of 2 x 44 units from C-2&18 positions). The ion at *m/z* 431 was similarly derived from the loss of CH_3 radical by the ion at m/z 445 followed by protonation (445-15+1=431, Figure 5C) [1]. The last compound detected in the porphyrin mixture (tret. 11.4 min) with *m/z* 591 value in the full MS-spectrum (Figure 3D) assigned as methyl-derivative of the protoporphyrin IX was shown similar to MS/MS spectrum of the porphyrins discussed above (Figure 5D).

Conclusion

The application of powerful UHPLC chromatography in the combination with DAD and ESI MS detections was presented in this paper. Five porphyrin derivatives: hematoporphyrin IX, protoporphyrin IX, two vinyl-hydroxyethyl isomers and methoxy isomer of protoporphyrin IX were identified in the hematoporphyrin derivatives mixture. Since hematoporphyrin and its derivatives are widely used porphyrin-photosensitizers in pharmacy, medicine etc., this research could be considered as the initial step in the analysis and

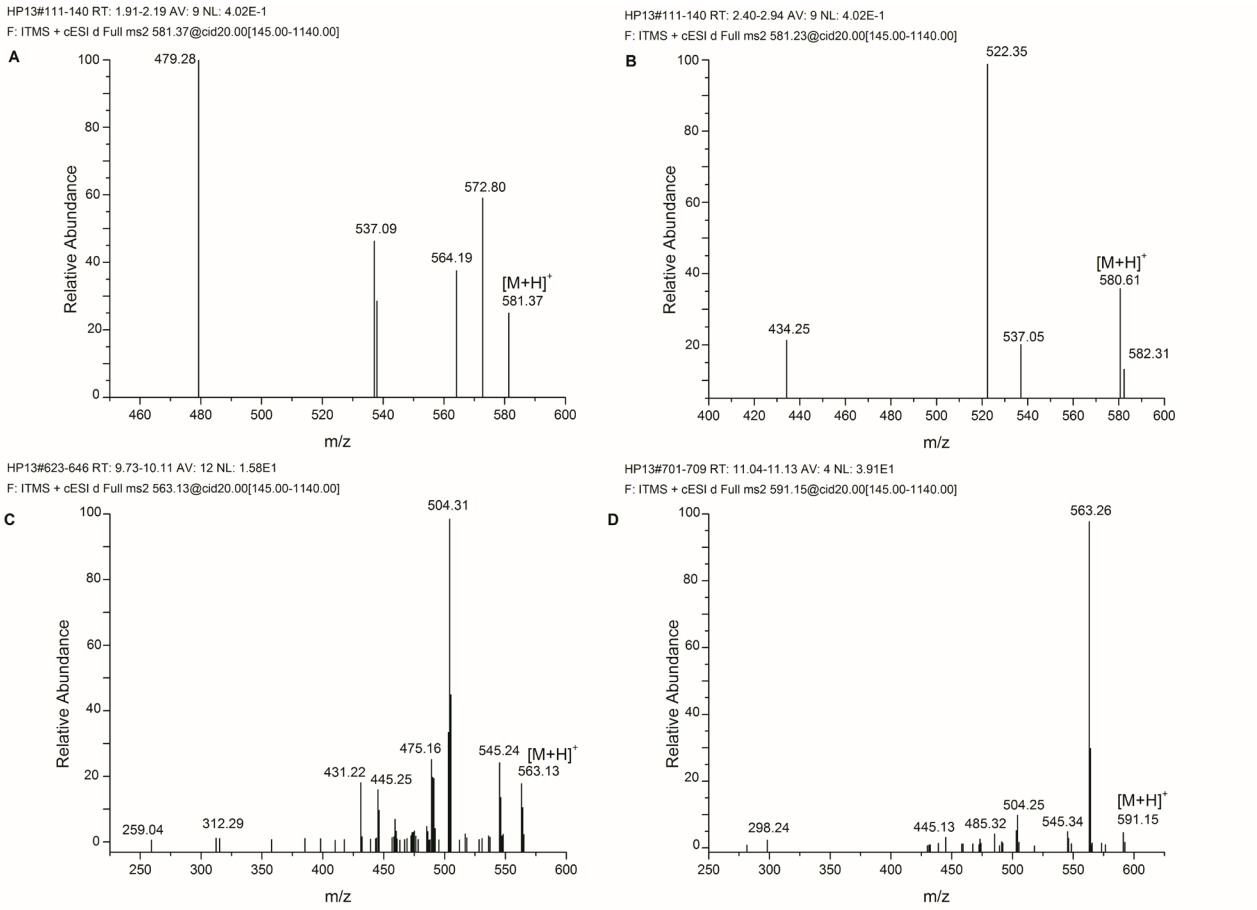

Figure 5. MS/MS spectra of vinyl-hydroxyethyl isomer I (A), vinyl-hydroxyethyl isomer II (B), protoporphyrin IX (C) and methyl-ester of protoporphyrin IX (D).

development of their further application.

Acknowledgements

This work was supported under the Project on Development of Technology number TR-34012 by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Izvod

PRIMENA HROMATOGRAFIJE VISOKIH PERFORMANSI KOMBINOVANE SA DAD I MS DETEKCIJOM U ANALIZI DERIVATA HEMATOPORFIRINA

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UDK 612.461.27:543.51:543.544

Nedavni napredak u tehnologiji kolona za visokoefikasne hromatografske metode doveo je do razvoja malih čestica kao nosača stacionarne faze u reverznofaznoj tečnoj hromatografiji, u analizi širokog spektra molekula. Ovaj rad predstavlja primenu UHPLC hromatografije (tečna hromatografija veoma visokih performansi sa C-18 česticama veličine 1.9 μm) kombinovane sa ESI-MS (elektrosprej jonizacija-masena spektrometrija) i DAD (detektor umreženih fotodioda) analizom za identifikaciju smeše porfirina poput hematoporfirina i protoporfirina, kao i njihovih derivata koji se često primenjuju kao fotosenzibilizatori u medicini. Pet porfirinskih derivata je razdvojeno i identifikovano u hematoporfirinskoj smeši: hematoporfirin IX, protoporfirin IX, dva hidroksietil-vinil-derivata i metil-estar protoporfirina IX.

Ključne reči: hematoporfirin IX; protoporfirin IX; tečna hromatografija; masena spektrometrija