Summary of Ph.D. thesis

Study of biologically relevant compounds by Mass Spectrometry

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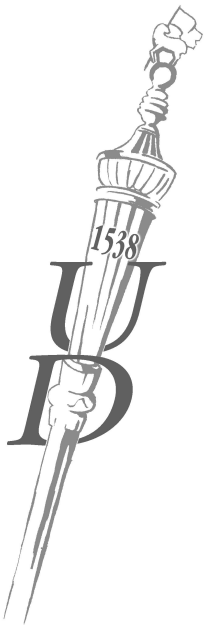

University of Debrecen
Chemistry Graduate School
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I. INTRODUCTION AND AIMS

Mass spectrometry (MS) is one of the most widely used analytical methods with high sensitivity and accuracy.

Mass spectrometry can be utilized for the direct analysis of both pure materials and mixtures. Even large molecules can be ionized without fragmentation thanks to soft ionization methods. High accuracy mass-to-charge (m/z) measurements allow not only the determination of molecular mass but even that of molecular the formula of the analyte. However, detailed structural information can only be derived from tandem MS (MS/MS) measurements. In case of the instrument is equipped with two analyzers, the parent ion selected by the first mass analyzer is fragmented e.g. in a collision cell and the fragments are separated by a second mass analyzer. Tandem MS spectra recorded under similar conditions can be used for fingerprint identification of molecules.

One of the leading modern analytical methods is liquid chromatography coupled mass spectrometry (LC-MS). Measurements obtained by LC-MS convey new levels of information compared to those obtained by the corresponding single techniques. This coupled method can be used for quantitative analysis thanks to the chromatographic separation. When the identity of the analyte is not unambiguous because of co-elution and/or identical molecular mass, LC can be combined with MS/MS to obtain structural information. To ensure selectivity, even the signal of a characteristic fragment ion can be monitored by MS/MS which makes this one of the most sensitive chromatographic detectors.

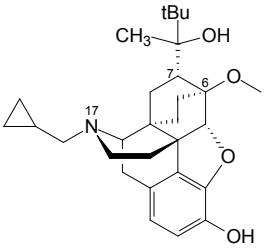
However, it should be kept in mind that structural information can be obtained by MS/MS only if the fragmentation properties of the target molecules are well characterized. The aim of my graduate studies was to investigate the MS/MS fragmentation properties of some selected biologically active compounds.

One group of targets consists of buprenorphine and 4 of its synthetic precursors which differ only in the substitution of the core

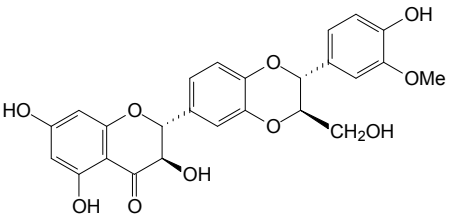
ring system. Buprenorphine is a semi-synthetic opioid receptor agonist, antagonist. Its mechanism of action is similar to that of morphine, but without the risk of addiction. In higher dosage buprenorphine is used to treat morphine addiction, in lower dosage it is administered as an effective painkiller.

A new method was developed in our research group for the extraction of active ingredients from milk thistle (*Silybum marianum*) seed. The active extract is known as silymarin, which is a mixture of silybin (A and B) and isosilybin (A and B) compounds, contains minor amounts of other constitutional isomers, such as silychristin A and B and silydianin, and also contains stereoisomers of the previous. An LC-MS/MS method was previously developed for the separation of silymarin components and their fragmentation properties were also studied in our research group. The aim of the investigation presented in the thesis was to study the energy dependence of the fragmentation of silymarin components and differentiate between fragmentation patterns of structurally closely similar components. Silymarin is used as dietary supplement because of its liver protective and liver regenerative properties. The anti-tumor properties of silybin are also proved.

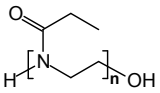
The third part of this thesis is centered on the characterization of synthetic poly(2-ethyl-2-oxazoline). The fragmentation of positive polymer ions are detailed under electrospray ionization (ESI) conditions. Poly(2ethyl-2-oxazoline) is produced in living cationic polymerization, thus its polymerization state is well tunable. The polymer is biocompatible and heat sensitive which gives rise to several medical applications e.g. as a carrier for active pharmaceutical ingredients.



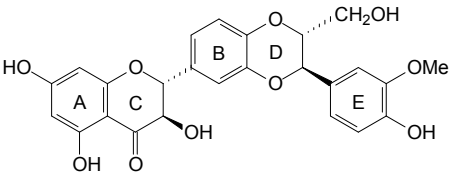
Buprenorphine



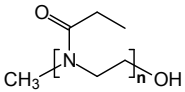
Silybin A



H-pEtOx



Isosilybin A



Me-pEtOx

Scheme 1 Structure of the studied compounds.

II. EXPERIMENTAL METHODS

The fragmentation of all studied compounds was studied with a Bruker micrOTOF$_Q$ mass spectrometer equipped with quadrupole and time-of-flight (Q-TOF) analyzers. Atmospheric pressure ionization techniques was used i.e. ESI (electrospray ionization), APCI (atmospheric pressure chemical ionization) and DART (direct analysis in real time). MS/MS experiments were performed using the first quadrupole analyzer to select the precursor ion, which was accelerated and then dissociated in the collision cell with N_2 gas. The fragment ions formed from the precursor ion were separated by the second TOF analyzer and the exact mass of these ions were determined. All the obtained MS and MS/MS spectra were evaluated by the DataAnalysis 3.4 software from Bruker.

In pseudo MS^3 experiments a fragment ion with a previously determined m/z value were generated in the ion source. This fragment ion was selected as a precursor ion with the first analyzer and then dissociated in the collision cell. With this method the further fragmentation of the selected fragment ion can be investigated.

The energy dependence of the dissociation was also studied and the results were interpreted by breakdown curves. These curves show the intensity of the fragment ion in the MS/MS spectra versus the applied collision energy. The appearance energy of the fragment ion and the collision energy at which the fragment has the maximum intensity can be determined.

The buprenorphine and derivatives samples were introduced directly to the mass spectrometer since these compounds were available individually. The silymarin sample was a mixture of active substances with the same elemental formula and the same molecular mass. Thus fragmentation of these compounds can be studied using mass spectrometry coupled with a separation technique. High performance liquid chromatography (HPLC) was used with diode array detector (Waters W2695 Separation Module, W2996 DAD detector). The post column flow was directed to the mass spectrometer

via a splitter, which allows the eluent to go partially into the ionsource to increase the efficiency of the ionization.

The pEtOx polymer was characterized before the fragmentation study. Mass spectrometry and size-exclusion chromatography was used for this purpose. With mass spectrometry not only the number average and mass average molecular mass can be determined but also the mass of the repeat unit and the end groups. MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometer) and Q-TOF MS with ESI, APCI and DART ionsources were used for the characterization of the polymer. Two pEtOx polymer series were identified with different end groups. The H-pEtOx has hydrogen and hydroxyl end groups, while the Me-pEtOx polymer has methyl and hydroxyl end groups. These polimer series were separated by HPLC (Waters W2695 Separation Module). The fragmentation behavior of the oligomers were studied and compared with different ionization conditions (ESI, APCI and DART) in a Q-TOF mass spectrometer.

III. SCIENTIFIC ACHIEVEMENTS

1. The collision induced dissociation (CID) of protonated buprenorphine and its synthethic precursors were studied by electrospray quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS)

1.1. The protonated buprenorphine formed in the ESI ionsource can be fragmented in the quadrupole collision cell. We found, that the fragmentation starts with the elimination of the substituent on the N together with the subsequent rupture of the piperidin ring. This process is parallel with the losses of small molecules from the substituent at C(7). The methoxy substituent at C(6) can also eliminate as methanol at lower collision energy. The breakdown curves were determined for each fragment ions appeared in the MS/MS spectra. These plots show the intensities of the ions versus the applied collision energy. Based on the appearance energy and the maximum intensity the order of formation of the ions can be suggested. The consecutive or parallel reactions can be realized.

1.2. Based on energy-dependent CID experiment we showed, that the molecules with acetyl substituent at C(7) have higher stability toward fragmentation, than the molecules with $–C(CH_3)(tBu)OH$ or $–C(CH_3)(Pr)OH$ substituents. We also showed, that these secondary alcohol substituents eliminate in two or three steps, and can not convert to acetyl group. Since at low collision energy the substituents at the N, and C(7) atoms already eliminates, the further dissociation occur from a common fragment ion in the cases of all the five studies compounds.

1.3. Increasing the collision energy the MS/MS spectra of all five studied compounds are similar, because their core-ring structures are the same (except the methoxy or hydroxy group at the aromatic ring). We proposed structure for most of the formed fragment ions based on the measured accurate mass. General fragmentation pathway of the core ring structure was also suggested. Pseudo MS^3 experiment were carried out to confirm the assumptions. In these experiment the

already observed fragment ions were generated in-source and the further dissociation of these ions were studied by MS/MS. We collected the breakdown curves of the fragment ions formed from the core-ring structures to a two dimensional plot (Figure 1).

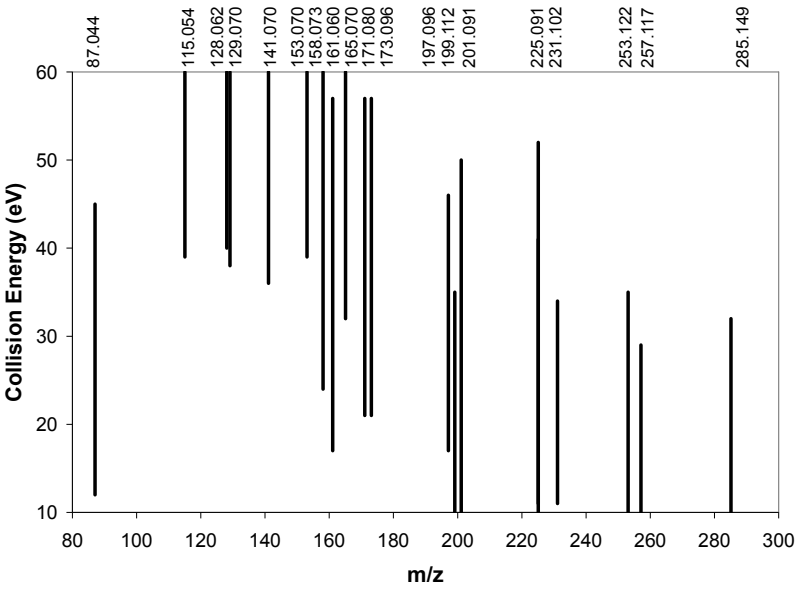


Figure 1 Presence of the fragment ions formed from the core-ring structure depending on the collision energy.

This plot shows the appearance energy of each ion, and the collision energy, at which they disappear from the MS/MS spectra. It also shows which fragment ions are present in the MS/MS spectra at a given collision energy.

1.4. The overall fragmentation patterns of buprenorphine and its synthetic precursors show several similarities to that of the related morphinans, and the main differences are due to the C(18)- C(19) ethylene bridge. Mechanism was proposed for the formation of the ethylene bridge related fragments.

2. Energy dependent CID study of the silymarin constituents. Interpretation of the distinct fragmentation behavior of two compounds with highly similar structure

2.1. Collision energy dependence of the fragmentation of the major silimarin constituents were investigated by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF). The survival yield (SY) plots were constructed for each components, which shows the SY depending on the applied collision energy. The SY value is equal with the ratio of the intensity of the precursor ion and the sum of the intensities of the fragment ions present in the MS/MS spectra. The survival yield (SY) plots of silydianin, the silybins and the isosilybins coincide with each other, while the SY plots of the silychristins are shifted to lower energies with about 5 eV. The CE_{50} energy values were determined for each components, these show the collision energy needed for 50% fragmentation of the precursor ion. As the breakdown diagram analysis revealed it is advisable to record the MS/MS spectra of the components at adjusted laboratory frame collision energies to get the same SY value (e.g., 50%) in each case, as this technique ensures higher reproducibility, thus can be utilized for the rapid identification of the silymarin constituents.

2.2. We also highlighted and explained that a simple substituent exchange can dramatically alter the fragmentation properties as in the case of silybin A and isosilybin A. The breakdown curves of the fragment ions with different intensity can be seen in Figure 2.

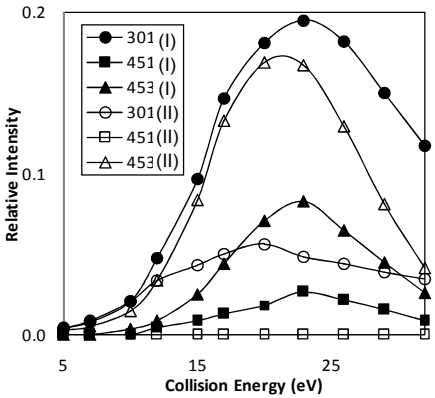
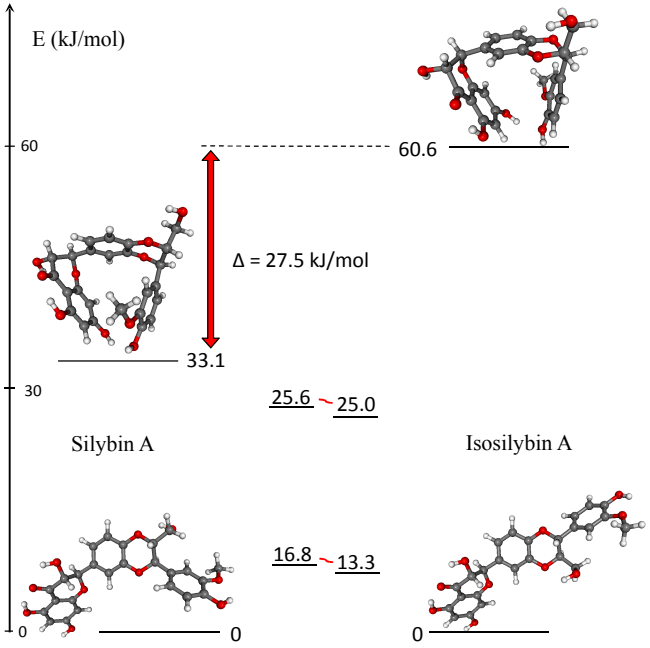


Figure 2 Breakdown diagrams for the fragment ions m/z 301, m/z 451 and m/z 453 of silybin A (I, filled points) and isosilybin A (II, empty points).

The fragment ion m/z 301 has higher intensity in the MS/MS spectra of silybin A, while the m/z 453 has higher intensity in the spectra of isosilybin A. Fragment ion m/z 451 appear only in the spectra of silybin A. The distinct fragmentation behavior is due to a stability shift between the possible conformations. The substituents on the rings C and D are equatorial in the most stable conformers of both compounds. Similar energy is needed to change the conformation of one of the rings from equatorial to axial. However the results of the conformational analysis show that the double diaxial conformer of silybin A has significantly lower relative energy compared to the analogous conformer of isosilybin A. The most stable conformers were optimated in the level of B3LYP/6-31G (d) and the relative energies were determined (Scheme 2). In the mass spectrometer it has higher possibility to have the double diaxial conformation is in the case of silybin.



Scheme 2 3D view of the most stable conformers of silybin A (left) and isosilybin A (right) in an energy plot.

3. Detailed fragmentation study of the poly(2-ethyl-2-oxazoline) polymer. Energy dependence of the dissociation steps and effect of number of repeat units on the characteristic collision energy.

3.1. The synthesized poly(2-ethyl-2-oxazoline) polymer was characterized by SEC, MALDI-TOF MS and Q-TOF MS equipped with ESI, APCI, DART ion sources. Two different polymer series appeared in the mass spectra because of chain transfer reactions during the polymerization (one with hydrogen and hydroxyl end-groups, and the other with methyl and hydroxyl end-groups).

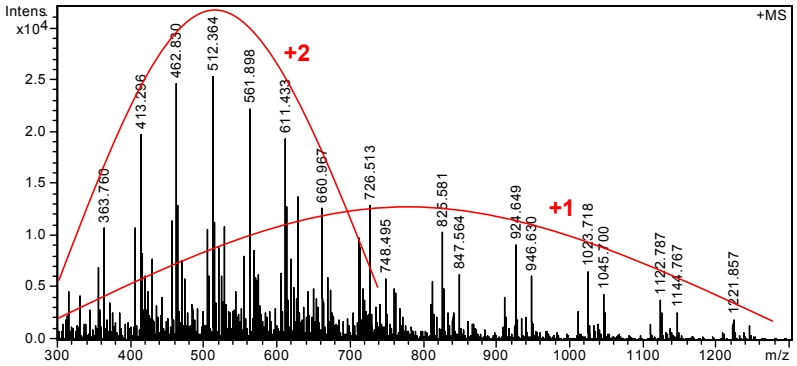
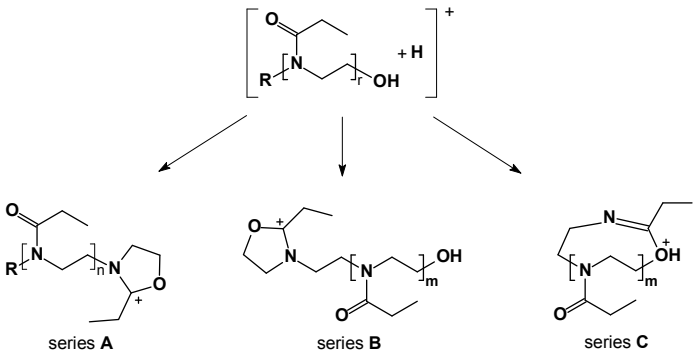


Figure 3 ESI Q-TOF MS spectrum of the poly(2-ethyl-2-oxazoline) polymer.

The average molecular weights and polydispersity were compared after determination by SEC, MALDI, APCI and ESI MS. The average molecular weight determined by APCI MS is lower than determined by the other techniques, because the fragmentation of oligomers with bigger molecular mass already starts in the high temperature ionsource. The two different polymer series can not be separated by size exclusion chromatography, however there existence was proved by reverse phase liquid chromatography.

3.2. The fragmentation behavior of the protonated oligomers was studied. Three product ion series formed from the precursor ion were identified (Scheme 3), and a mechanism was proposed for the dissociation, based on the accurate mass of the product ions, pseudo MS^3 experiments and the energy-dependence of the product ion intensity.



Scheme 3 Proposed structure of the three product ion series formed from pEtOx polymer

Two additional losses were observed in the MS spectra, the elimination of a side group and elimination of propionic acid within the chain. The results of the MS/MS experiments of the polymer dissolved in deuterated methanol confirmed the proposed mechanisms.

3.3. The survival yield method was used to describe the efficiency of the fragmentation quantitatively; and it was studied as a function of chain length of the pEtOx oligomer and the ionization method. Linear correlation was established between the number of the repeat units of the oligomers and the laboratory frame collision energy. This simple relationship enables the prediction of proper laboratory frame collision energy to obtain structural information for polymers.

IV. POSSIBLE APPLICATIONS OF THE RESULTS

Liquid chromatography coupled with mass spectrometry and tandem mass spectrometry are analytical methods which can be used for qualitative and quantitative purposes simultaneously thanks to their high sensitivity and accuracy. Understanding the studied fragmentation pathways is essential in pharmaceutical applications and also facilitates prognosis of fragmentation of related substances. Fragment ions common for a collection of related substances help categorize unknown analytes, and a series of specific fragments can unambiguously identify the structure of a parent molecule. The detection limit of a given analyte is the lowest when scanning for a selected fragment ion at a collision energy optimal for its formation. Choosing the right value for collision energy is essential for recording good quality MS/MS spectra, because at low energies fragment ions are scarce and at high energies there are too many fragments to clearly evaluate the spectrum. It is therefore important to know the energy dependent fragmentation pathways of given analytes especially when dealing with complicated analytical problems and low detection limits.

V. LIST OF PUBLICATIONS

Publications covered in the thesis

1. Energy-dependent collision-induced dissociation study of buprenorphine and its synthetic precursors
   B. Biri, J. Kalmár, L. Nagy, A. Sipos, M. Zsuga, S. Kéki
   Rapid Communications in Mass Spectrometry (2011), 25(1), 41-49.
   IF: 2,790

2. Collision induced dissociation study of the major components of silymarin
   Á. Kuki, B. Biri, L. Nagy, Gy. Deák, J. Kalmár, A. Mándi, M. Nagy, M. Zsuga, S. Kéki
   International Journal of Mass Spectrometry (2012), 315, 46
   IF: 2,549

3. Collision-induced dissociation study of poly(2-ethyl-2-oxazoline) using survival yields and breakdown curves
   B. Biri, L. Nagy, Á. Kuki, E.R. Tőke, Gy. Deák, M. Zsuga, S. Kéki
   Journal of Mass Spectrometry (2013), 48(1), 16-23.
   IF: 3,268

Σ IF=8,607

Articles not detailed in the thesis:

1. Photodecomposition of o-phthaldialdehyde-derivatized amino acids by the photodiode array detector during their high-performance liquid chromatographic analysis
   S. Kéki, J. Török, B. Biri, J. Zsuga, R. Gesztelyi, D. Bereczki, M. Zsuga
   Journal of Chromatography A (2008), 1185, 301. **IF: 3,756**

2. Increased production of asymmetric dimethylarginine (ADMA) in ankylosing spondylitis: Association with other clinical and laboratory parameters
   Á. Kemény-Beke, R. Gesztelyi, N. Bodnár, J. Zsuga, Gy. Kerekes, M. Zsuga, B. Biri, S. Kéki, P. Szodoray, A. Berta, Z. Szekanecz, S. Szántó
   Joint Bone Spine (2011), 78(2), 184. **IF: 2,274**

3. Systematic identification of active ingredients of Silybum Marianum seed
   L. Nagy, Á. Kuki, G. Deák, B. Biri, M. Nagy, M. Zsuga, S. Kéki
   Jurnal Medical Aradean (Arad Medical Journal) (2011), 14 (2), 9-12. **IF: -**

4. Mechanism of Decomposition of the Human Defense Factor Hypothiocyanite Near Physiological pH
   J. Kalmar, K. Woldegiorgis, B. Biri, M. Ashby
   Journal of the American Chemical Society (2011), 133 (49), 19911. **IF: 9,907**

5. Detailed Mechanism of the Autoxidation of N-hydroxyurea Catalyzed by an SOD mimic Mn(III) porphyrin: Formation of the Nitrosylated Mn(II) porphyrin as an Intermediate
   J. Kalmár, B. Biri, G. Lente, I. Bányai, A. Budimir, M. Biruš, I. Batinić-Haberle, I. Fábián
   Dalton Transaction (2012), 41, 11875-11884. **IF: 3,838**

6. Identification and characterization of microbial biofilm communities associated with corroded oil pipeline surfaces
   T. R. Lenhart, K. E. Duncan, I. B. Beech, J. A. Sunner, W. Smith, V. Bonifay, B. Biri, J. M. Suflita
   Biofouling közlésre beküldve

Σ IF=19,775

International and national conference attendance:

1. Methylarginine metabolism is different in acute and chronichypoxia:
   12AP1 – 3
   T. Molnár, B. Sütő, B. Biri, L. Nagy, S. Kéki, I. Ruzsics
   European Journal of Anaesthesiology **2013**; 30: 181.

2. Szénhidrogének anaerob biodegradációja során képződő metabolitok LC/MS vizsgálata (E)
   Biri B., D. Aktaz, J. Sunner, Kéki S.
   XVII. Nemzetközi Vegyészkonferencia, 2011. nov. 3-6., Kolozsvár.

3. A hipotiociános-sav (HOSCN) vizes oldatbeli bomlása fiziológiáshoz közeli pH-n (E)
   Kalmár J., Biri B., K. L. Woldegiorgis, M. T. Ashby
   XVII. Nemzetközi Vegyészkonferencia, 2011. nov. 3-6., Kolozsvár.

4. Egy nem azonosított Silymarin komponens elválasztása oszlopkromatográfiás módszerrel (E)
   Deák Gy., Biri B, Kuki Á., Nagy L., Zsuga M., Kéki S.
   XVII. Nemzetközi Vegyészkonferencia, 2011. nov. 3-6., Kolozsvár.

5. Mass spectrometric approaches to the study of bacterial metabolomes aimed at diagnosis of anaerobic hydrocarbon degradation and associated biocorrosion (P)
   J. Sunner, B. Biri, S. Foster, I. Beech, M. Kowalski, J. Suflita, D. Aktas, I. Davidova
   59th ASMS Conference on Mass Spectrometry and Allied Topics, June 5-9, 2011, Denver, Colorado, USA

6. A hipotiociános-sav (HOSCN) vizes oldatbeli bomlásának mechanizmusa fiziológiáshoz közeli pH-n (E)
   Kalmár J., Biri B., K. L. Woldegiorgis, M. T. Ashby
   MTA Reakciókinetikai és Fotokémiai Munkabizottság ülése, 2011. okt. 27-28., Gyöngyöstarján.

7. Zsírsav származékok mennyiségi meghatározása (E)
   Biri B., Oláh A., Sós K., Zsuga M., Kéki S.
   MTA Tudomány Napja, Doktoranduszok Fóruma, 2010. nov. 4., Debrecen

8. Tandem tömegspektrometria szerepe: Szilimarin komponensének APCI-MS/MS fragmentációs vizsgálata (E)
   B. Biri, L. Nagy, Á. Kuki, M. Zsuga, S. Kéki
   International Conference „Natural and artificial ecosystems in the Somes- Cris- Mures- Tisa river basin” May 7-8, 2010, Macea, Romania

9. Vérszérum aminosav tartalmának meghatározása (E)
   Biri B., Zsuga J., Gesztelyi R., Bereczki D., Zsuga M., Kéki S.
   XV. Nemzetközi Vegyészkonferencia, 2009. nov. 12-15., Marosvásárhely.

10. Anandamid és 2-Arachidonoil-glicerin meghatározása APCI-MS módszerrel (E)
    Biri B., Kéki S.
    MTA Műanyag és Természetes Polimerek Munkabizottság: munkabizottsági ülés, 2009. ápr. 23., Budapest

11. Anandamid és 2-Arachidonoyl-glicerin kvantitatív meghatározása sejtkultúrákból (E)
    Biri B., Kuki Á., Nagy L., Kéki S., Zsuga M., Ambrus L., Lisztes E.
    XIV. Nemzetközi Vegyészkonferencia, 2008. nov. 13-15., Kolozsvár

(E: Presentation, P: Poster)