Short thesis for the degree of doctor of philosophy (PhD)

The development of microfluidic immobilized enzymatic reactors for proteomic applications

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I. Introduction and objectives

Proteomic studies aim at the comprehensive characterization of the proteome. The field of analytical chemistry offers a wide range of techniques for protome mapping, where each method provides complementary pieces of information. Usually, the most burning question is what proteins are present in the sample (regarding their post-translational modifications, as well) and in what quantity. Analytical platforms utilizing a separation technique coupled to mass spectrometry (MS) are highly adept at answering this question. Depending on whether the proteins are analyzed in their intact, partially or completely hydrolyzed form, we can distinguish top-down, middle down/up and bottom-up proteomics. From among the three approaches, the bottom-up strategy has the most solid history, however, top-down proteomics is also gaining popularity, due to the improvement of mass analyzers and the application of alternative fragmentation techniques.

In bottom-up proteomics – as the name suggests – smaller peptides are pieced together to acquire the ”whole picture”. The hydrolysis of proteins is usually carried out by a protease (either one or more if necessary). The past couple of decades have seen the streghtening emergence of miniaturization trends, which have penetrated the field of proteomic method development, as well, targeting especially the optimization of proteolysis. In these microfluidic devices the enzymes are most often confined to a solid support. Such systems are called microfluidic immobilized enzymatic reactors (μ-IMER). Contrary to the standard digestion procedure performed in solution phase where the enzyme is present in its free form, the immobilized enzymes demonstrate reusability and minimal autolysis and so can retain their activity longer. Furthermore, the elevated surface area-to-voume ratio of the microreactors increases the enzyme-substrate ratio considerably, leading to a significant acceleration of reaction times. Owing to the advantages listed above, there is a growing interest in the development and proteomic application of μ-IMERs; nearly a hundred papers were published annually in the past years about such microreactors.

When dealing with real samples it is often the case that the proteins are present only in low concentrations while contaminants can have a significant contribution to the overall sample composition. Prior to analysis; therefore, it is strongly recommended to include a sample clean-up and preconcentration step (e.g., solid-phase extraction – SPE), whereby the target analytes are transferred into a considerably smaller volume. SPE is often used for the desalination of samples in the field of bottom-up proteomics, where the interefering components from the samples can be eliminated easily. In certain cases; however, only limited sample volumes are available. In capillary electrophoresis (CE) systems, there is also a possibility for on-line preconcentration, which can be performed in the same analytical unit (capillary) and so sample loss can be avoided. Sample preconcentration techniques include field-assisted sample stacking (FASS), (transient) isotachophoresis ((t)-ITP) or dynamic pH junction (DPJ).

During my PhD years two main objectives were formulated. The first was to design easily manufacturable μ-IMER systems that are capable of accelerated proteolysis (~ min). The second objective was focused on investigating the issue of desalination and in connection with this, the development of a CE-MS method utilizing on-line preconcentration for increasing the peptide and protein hits.

II. Methods

Microfluidic chips were made of polydimethylsiloxane (PDMS) by means of soft lithography. Microchannels were designed using AutoCAD software (v.2013, Autodesk, San Rafael, CA, USA). Two types of channel structures were designed. One is a wider channel (w: 375 μm) including pillar obstructions in an ordered arrangement, the other is a narrower (w: 25-75 μm) serpentine-like channel. Trypsin was immobilized to the PDMS surface by spontaneous adsorption. Freshly prepared trypsin solution (20 mg/mL) was pumped through the channel (2 μL/min, 10 min). Unbound trypsin was eliminated by flushing the channel with 25 mM NH_4HCO_3 solution (2 μL/min,

10 min). A peristaltic pump (IPC, Ismatec, Cole-Palmer, IL, USA) was used for the transportation of liquids through the channels. The pump tubing (inner diameter: 0.19 mm, Tygon, Cole-Palmer, IL, USA) was connected to the inlet port of the channel in order to establish fluid connections. In the case of the in-line IMER-CE-MS system, trypsin was immobilized to the initial ~ 2 cm of the fused silica capillary by electrostatic interactions.

The performance of the microreactors was evaluated by digesting different protein samples (human serum albumin – HSA, snake venom, human tear and saliva) and comparing the efficiencies with that of in-solution digestion. For sample pretreatment, the following excipients were used: urea, for disrupting the tertiary structure of the proteins; dithiothreitol (DTT), for reducing the disulfide bonds and iodoacetamide (IAM) for preventing the recombination of the disulfide bonds by alkylating the SH-groups formed. Finally, samples were diluted with 25 mM NH_4HCO_3 solution in order to reduce the urea concentration to below ~ 1 M (so as to preserve trypsin activity) and to ensure optimal pH for tryptic digestion. The pretreated samples were transported through the reactors for IMER-based digestions. In the case of in-solution digestion, trypsin solution was added to the mixture (trypsin:protein = 1:50) and after a 16-hour long incubation, the reaction was quenched by adding 1% formic acid (FA) to the solution.

The digests were analyzed with capillary zone electrophoresis (CZE) using UV and mass spectrometric (MS) detection. CZE measurements were conducted with an Agilent 7100 CE instrument (Agilent, Waldbronn, Germany). UV detection was carried out at λ= 200 nm (bandwidth: 4 nm, no reference, response time: 0.3 s). For MS detection a maXis II qTOF-MS (Bruker, Bremen, Germany) was used. The CE and MS instruments were on-line coupled with a CE-ESI Sprayer (G1607B, Agilent) interface. The sheath liquid was transported by a 1260 Infinity II isocratic pump (Agilent). The sheath liquid consisted of isopropanol:water = 1:1 + 0,1 % FA and was pumped at a flow rate of 4 μL/min volt. The fused silica capillaries used for measurements were usually 90 cm long (inner diameter: 50 μm) (Polymicro, Phoenix, AZ, USA). Hydrodynamic sample introduction was carried out at the anodic end of

the capillary (positive polarity). The background electrolyte (BGE) was usually 1 M FA (pH= 1.9). Applied voltage was typically +25-30 kV, temperature was set to 25 °C. Parameters set for MS detection: positive ionization mode; m/z range: 50-2200; MS spectra rate 3 Hz; MS/MS spectra rate 1-4 Hz (depending on precursor ion intensity). Fragments were generated by collision-induced dissociation (CID).

Liquid flow in microchannels was investigated by COMSOL Multiphysics software (Burlington, MA, USA) (ver.: 5.3), the operation of which is based on the finite element method (FEM).

III. New scientific results

1. For the first time, a pillar array microchannel with enhanced specific surface area was formed in a PDMS microchip for IMER-related applications.

1.1 It was determined that the limit of miniaturization with the soft lithographic microfabrication technique is ~25-30 μm.

Our objective was to increase the surface area-to-volume ratio (S/V) of microfluidic chips the easiest way possible. Since the reactor has an open tubular layout (there is no subsequently introduced S/V enhancing medium, e.g., porous layer, monolith or silica particles), the goal was to increase the specific surface area with geometric features: pillars were created in the microchannel by the soft lithographic technique we use for microfabrication. Minimizing the pillar diameter and interpillar distances favours high S/V, however, our soft lithographic technique imposes some limitations on miniaturization. The microfabrication procedure consists of the main steps: first we develop the mold using photolithography, by which the designed pattern on a lithographic mask is transferred to a silicon wafer covered by a photosensitive resist; then replicas are made from this template using PDMS. During the optimization of the microfabrication process we determined that the limit of miniaturization for us

is ~ 25-30 µm (Figure 1). Channels containing pillars of such dimension enable a significant boost is specific surface area.

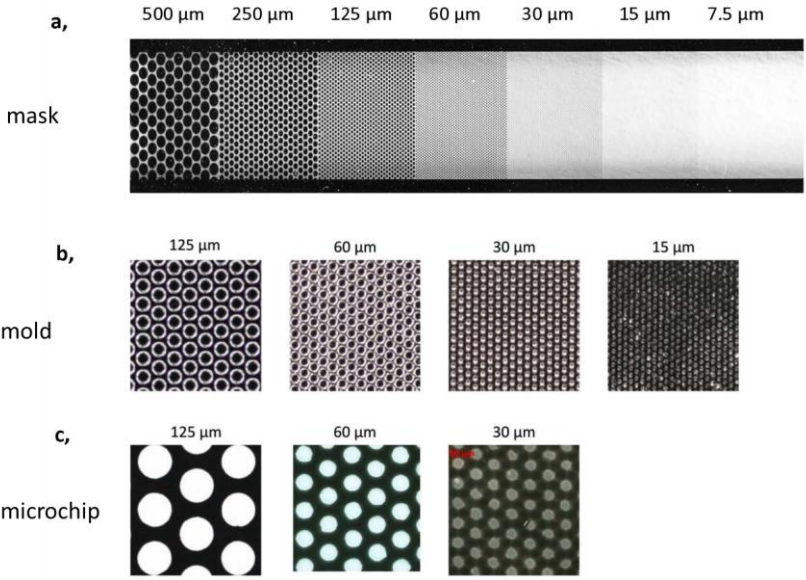

Figure 1: (a) the lithographic mask containing pillars of decreasing size, (b) zoomed in images of the relevant sections of the mold created and (c) images of the microfluidic chips.

Although in the literature we can find works where pillar diameters as low as 5 µm were used, the formation of these structure is typically carried out with dry etching microfabrication techniques, which requires expensive instrumentation and special infrastructure (e.g., cleanroom).

1.2 We compared the digestion efficiencies of the developed IMERs with that of in-solution digestion. Peak patterns observed in CZE peptide maps as well as identified proteins show high conformity, even though the reaction time in IMER-based digestions was reduced to ~ 1 min (from the 16 hour long digestion typical of in-solution digestion).

Protein samples of differing complexity (human serum albumin – HSA, snake venom) were digested with the microreactor developed. CZE-UV peptide

maps showed great similarity to those of in-solution digestion. (Figure 2). No signal indicating the presence of undigested protein (broad, tailed peaks) can be seen in the peak patterns. CE-MS/MS analyses also confirmed the reliability of the IMER; a good agreement was observed between IMER and in-solution-based digestions in terms of peptide and protein hits (~80% sequence coverage could be achieved for HSA with both methods).

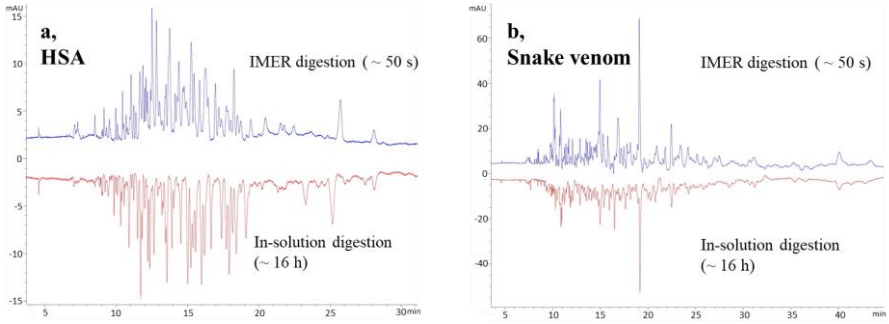

Figure 2: The comparison of CZE-UV peptide maps obtained with IMER-based and in-solution digestion for (a) HSA and (b) snake venom samples.

2. It was verified that hollow channels of narrow inner diameter (< 100 µm), and of extended length (> 20 cm) are also amenable for use as enzymatic reactors, owing to their high specific surface area.

2.1 We determined that there is no point in decreasing channel diameters to below 25 µm, or increasing to above 100 µm, since there is considerable backpressure in too narrow channels, while the application of too wide channels might reduce the frequency of enzyme-substrate interactions.

The specific surface area of empty channels can also be increased by miniaturization. Since the enzymes are bound to the walls of the channels, decreasing the channel width causes a reduction in diffusion path length, as well, which favours the occurrence of enzyme-substrate interactions. We demonstrated that in a 100 µm wide channel it takes 20 s for an albumin molecule to diffuse to the wall of the channel from the center, while the same

process takes only ~1 s if channel width is reduced to 25 μm. However, such narrow channels have significant backpressure. Assuming a 20 cm long channel, the time required to transport 10 μL sample through the reactor is more than 10 hours in a 10 μm wide channel, however, it takes only ~15 minutes if the channel width is 25 μm. Based on the considerations above we concluded that the application of channel widths in the range of 25-75 μm is optimal.

2.2 Computer simulations proved that despite the strongly laminar flow conditions (Re<1) prevailing in very narrow channels (25-100 um), the incorporation of curvatures in the microchannels facilitates the passive mixing of the components in the solution phase.

In microfluidic channels, liquid flow is usually strongly laminar (streamlines are parallel). The simplest way to achieve the mixing of fluid layers is by incorporating passive mixers. Curvatures can be considered passive mixers, since they generate a secondary fluid flow (Dean vortices) and thereby increase the diffusion surface. There is no real consensus in the literature about the extent to which curves contribute to the mixing, but it has been shown that the Dean effect is most significant in systems where Re>10. We confirmed through COMSOL simulations that despite the strong laminarity (Re<1) in the system, curvatures do have a favourable impact on mixing (Figure 3). The first curvature has the largest contribution, while the effect of the last curve is only 10% compared to the first turn.

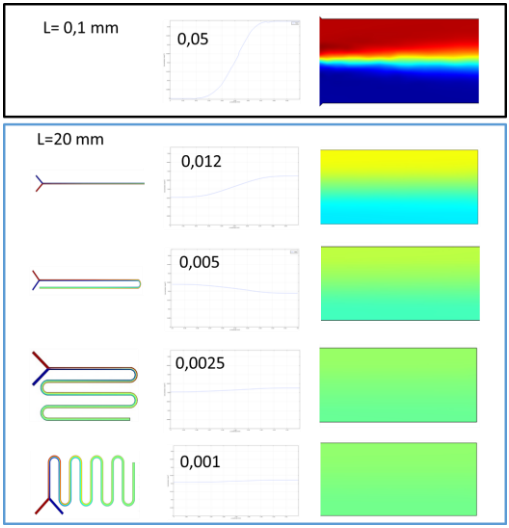

Figure 3: The effect of curvatures on the mixing efficiency. Top panel shows the initial concentration distribution (L = 0.1 mm), lower panel shows the final concentration distributions (L = 20 mm) for the four channels differing only in the number of incorporated curvatures: 0, 1, 4 and 8 curves.

2.3 In order to increase the applicable overall volumetic flow rate and hence, decrease digestion times, we designed channel structures where the liquid flow is split to 8 or 32 parallel channels at the inlet port.

The contact time was increased in the microreactors by designing split-flow architectures, where 8 or 32 parallel serpentine channels were connected to one inlet port. In the latter case, the flow was first split to 4 and then additional 8-8 channels. The advantage of split-flow is that the substrate solution comes into contact with the immobilized enzyme over a larger surface area, even when a higher overall volumetric flow rate is applied.

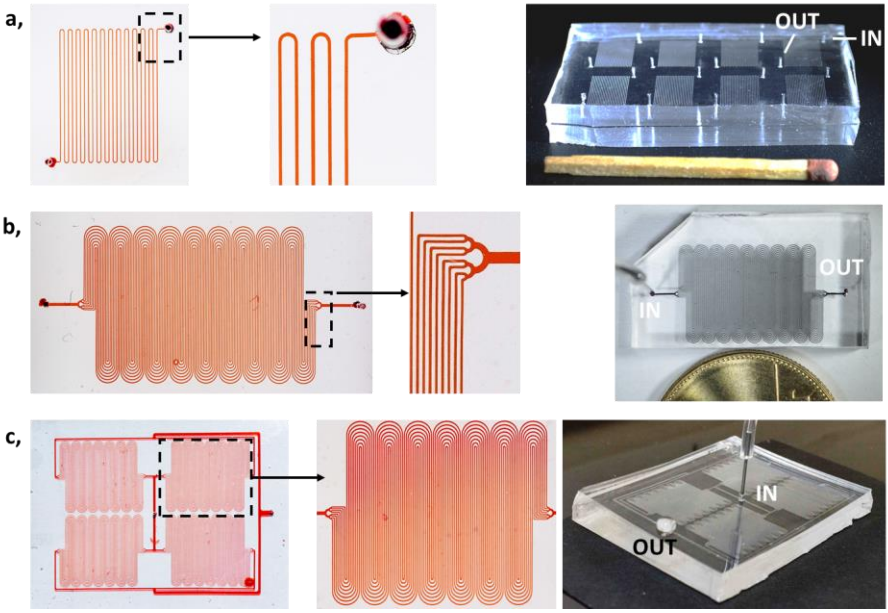

Figure 4: PDMS microchips having different channel systems (right). Images of the corresponding channels filled with red food dye (left). Channel parameters: ID = 25 µm; L = 20 cm; number of parallel channels: (a) 1; (b) 8 and (c) 32.

3. We developed an in-line IMER-CE-MS system, in which the immobilization, digestion, separation and detection steps are all carried out in a fully automated way. The microreactor section was formed at the initial part (~ 2 cm) of the separation capillary.

For online and especially in-line systems, it is important to harmonize the experimental conditions such that they should allow the proper functioning of all units in the workflow. In our case, the immobilization, digestion, and separation were all carried out in the same analytical unit (fused silica capillary). Trypsin was attached by adsorption only to the initial section of the capillary (~2 cm). Due to its relatively high isoelectric point (pI ~ 10.3), trypsin has a net positive charge in a wide pH range, and so it readily adsorbs to the negatively charged surface of the capillary (silanol groups deprotonate at pH ~4). The

optimal pH for trypsin activity (pH ~8) was ensured during the digestion. Since MS detection was performed, the volatility of the separation buffer had to be ensured, as well. NH_4Ac solution (40 mM, pH = 8.0) met all of these conditions. The in-line system was used for the digestion of HSA and human tear samples. Peptide and protein hits were consistent with those of in-solution digestion.

4. We showed that the sample matrix of protein digests are inherently suitable for inducing on-line sample enrichment during CE runs, rendering the commonly utilized sample clean-up procedures (e.g., SPE, buffer exchange) unnecessary.

4.1 It was confirmed that that high salt content (NH_4HCO_3) of the protein digests generates a t-ITP effect during the zone electrophoretic separations.

In the bottom-up workflow, digests are often desalinated prior to analysis in order to remove matrix components. When larger sample volumes are injected for CZE analysis, different on-line sample enrichment mechanisms may occur depending on the composition of the sample matrix. The analysis of HSA digests was performed to investigate this phenomenon. In the case of desalinated samples, field-assisted sample stacking (FASS) was the main mechanism for sample enrichment, while in non-desalinated samples, mainly transient isotachophoresis (t-ITP) was responsible for sample enrichment. During the sample preparation used in bottom-up analysis, significant amounts of NH_4HCO_3 are added to the samples, whereby the NH_4^+ ions can behave as leading ions, causing a t-ITP event. Peak intensities increased proportionally with the sample volume injected, while peak broadening was only marginal for most peptides.

4.2 We demonstrated that the commonly utilized SPE desalination of protein digests offers no advantages in the case of CE-MS peptide mapping.

Due to the sample preparation procedure, protein digests have a high salt content, which usually interferes with the analysis. The elimination of salts is commonly carried out with SPE desalination units. Peptide hits obtained from

the CE-MS measurement of desalinated and non-desalinated samples were compared. Peptides bound to the SPE C18 packing were eluted with mixture containing ACN:water = 70:30 + 0.1% FA. Due to the significant amount of organic solvent introduced into the sample, a FASS type sample enrichment mechanism was induced during the analysis. Peak intensities were very similar to those obtained by the analysis of non-desalinated digests, since sample enrichment occurred in both cases (the main mechanisms of which are different). However, a considerable difference was observed in the number of identified peptides (Figure 5). Desalination may result in peptide loss because small, hydrophilic peptides do not bind to the C18 phase and so are removed together with the salts. The best results were obtained with the third sample type (non-desalinated + ACN), which presumably benefits from the combined effects of t-ITP and FASS mechanisms due to the presence of salts and organic solvents in the sample.

	Normal injection		Stacking injection	
	#unique peptides	SC%	#unique peptides	SC%
desal.	15	25	32	43
non-desal.	11	20	43	48
non-desal. (+ ACN)	13	22	54	57

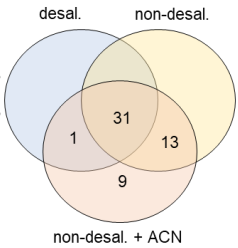

Figure 5: Results obtained for the three sample types: (a) sequence coverage values (SC %) and number of unique peptides for normal and stacking injections and (b) Venn-diagram, showing the number of identified peptides for each digest, highlighting the mutual peptides. Analyses were performed in quintuplicate.

IV. Potential applications of the results

The aim of proteomic research is the extensive characterization of protein samples. Depending on the approach used (top-down, middle-down/up or bottom-up), different information can be obtained, which can complement each other to form a more complete picture of the protein sample. Bottom-up proteomics is routinely used for the identification and verification of protein sequences, as well as for the qualitative and quantitative characterization of biological samples. It is increasingly important in the field of drug analysis due to the uptake of protein-based therapeutics. The bottom-up strategy is based on the enzymatic digestion of proteins, which is usually the rate-limiting step of the process (2-24 hours). The development of methods that can substantially accelerate this step is highly desired. Immobilized enzyme reactors can ensure the efficiency achieved by in-solution proteolysis (16 hours) in just a few minutes. The main advantage of these reactors is that the immobilization of trypsin does not require the use of derivatization reagents, the enzyme solution simply needs to be brought into contact with the carrier medium, making it easily regenerable. The use of the in-line IMER system is a fully automated workflow.

In addition to proteolysis, the analysis of the resulting peptides is also of paramount importance. The removal of salts and contaminants from the digests is often performed by SPE (which is also suitable for the enrichment of peptides, if the bound peptides are eluted into a smaller volume). It was shown that the salt content of the sample does not interfere with the CZE analysis; in fact, the salt content induces on-line sample preconcentration. Omitting the desalinating step is also advantageous in terms of sample integrity. Peptide loss resulting from SPE can be avoided, which enables the identification of more peptides/proteins.

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Registry number: DEENK/145/2023.PL
Subject: PhD Publication List

Candidate: Cynthia Nóra Nagy
Doctoral School: Doctoral School of Chemistry
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List of publications related to the dissertation

Foreign language scientific articles in international journals (4)

1. **Nagy, C.**, Szabó, R., Gáspár, A.: Microfluidic Immobilized Enzymatic Reactors for Proteomic Analyses-Recent Developments and Trends (2017-2021).
   Micromachines. 13 (2), 1-19, 2022. EISSN: 2072-666X.
   DOI: https://doi.org/10.3390/mi13020311
   IF: 3.523 (2021)

2. **Nagy, C.**, Szabó, R., Gáspár, A.: Development of an In-Line Enzyme Reactor Integrated into a Capillary Electrophoresis System.
   Molecules. 26 (19), 1-14, 2021. ISSN: 1420-3049.
   DOI: http://dx.doi.org/10.3390/molecules26195902
   IF: 4.927

3. **Nagy, C.**, Huszánk, R., Gáspár, A.: Study of the geometry of open channels in a layer-bed-type microfluidic immobilized enzyme reactor.
   Anal. Bioanal. Chem. 413 (25), 6321-6332, 2021. ISSN: 1618-2642.
   DOI: http://dx.doi.org/10.1007/s00216-021-03588-x
   IF: 4.478

4. **Nagy, C.**, Kecskeméti, Á., Gáspár, A.: Fabrication of immobilized enzyme reactors with pillar arrays into polydimethylsiloxane microchip.
   Anal. Chim. Acta. 1108, 70-78, 2020. ISSN: 0003-2670.
   DOI: http://dx.doi.org/10.1016/j.aca.2020.02.048
   IF: 6.558

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List of other publications

Foreign language scientific articles in international journals (9)

5. Andrási, M., Gyémánt, G., Sajtos, Z., **Nagy, C.**: Analysis of Sugars in Honey Samples by Capillary Zone Electrophoresis Using Fluorescence Detection.
   Separations. 10 (3), 1-10-, 2023. EISSN: 2297-8739.
   DOI: http://dx.doi.org/10.3390/separations10030150
   IF: 3.344 (2021)

6. Hamidli, N., Pajaziti, B., Andrási, M., **Nagy, C.**, Gáspár, A.: Determination of human insulin and its six therapeutic analogues by capillary electrophoresis - mass spectrometry.
   J. Chromatogr. A. 1678 (16), 1-8, 2022. ISSN: 0021-9673.
   DOI: https://doi.org/10.1016/j.chroma.2022.463351
   IF: 4.601 (2021)

7. Borrego, J., Naseem, M. U., Sehgal, A. N. A., Panda, L. R., Shakeel, K., Gáspár, A., **Nagy, C.**, Varga, Z., Panyi, G.: Recombinant Expression in Pichia pastoris System of Three Potent Kv1.3 Channel Blockers: Vm24, Anuroctoxin, and Ts6.
   J. Fungi. 8 (11), 1-15, 2022. EISSN: 2309-608X.
   DOI: http://dx.doi.org/10.3390/jof8111215
   IF: 5.724 (2021)

8. **Nagy, C.**, Andrási, M., Hamidli, N., Gyémánt, G., Gáspár, A.: Top-down proteomic analysis of monoclonal antibodies by capillary zone electrophoresis-mass spectrometry.
   Journal of Chromatography Open. 2, 1-21, 2022. EISSN: 2772-3917.
   DOI: https://doi.org/10.1016/j.jcoa.2021.100024

9. Hamidli, N., Andrási, M., **Nagy, C.**, Gáspár, A.: Analysis of intact proteins with capillary zone electrophoresis coupled to mass spectromery using uncoated and coated capillaries.
   J. Chromatogr. A. 1654, 1-9, 2021. ISSN: 0021-9673.
   DOI: http://dx.doi.org/10.1016/j.chroma.2021.462448
   IF: 4.601

10. **Nagy, C.**, Pesti, A., Andrási, M., Vasas, G., Gáspár, A.: Determination of artemisinin and its analogs in Artemisia annua extracts by capillary electrophoresis: Mass spectrometry.
    J. Pharm. Biomed. Anal. 202, 1-7, 2021. ISSN: 0731-7085.
    DOI: http://dx.doi.org/10.1016/j.jpba.2021.114131
    IF: 3.571

11. Kecskeméti, Á., **Nagy, C.**, Biró, P., Szabó, Z., Pócsi, I., Bartók, T., Gáspár, A.: Analysis of fumonisin mycotoxins with capillary electrophoresis: mass spectrometry.
    Food Addit. Contam. Part A-Chem. 37 (9), 1553-1563, 2020. ISSN: 1944-0049.
    DOI: http://dx.doi.org/10.1080/19440049.2020.1778797
    IF: 3.057

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12. Andrási, M., Pajaziti, B., Sipos, B., **Nagy, C.**, Hamidli, N., Gáspár, A.: Determination of deamidated isoforms of human insulin using capillary electrophoresis.
J. Chromatogr. A. 1626, 1-8, 2020. ISSN: 0021-9673.
DOI: http://dx.doi.org/10.1016/j.chroma.2020.461344
IF: 4.759

13. Kecskeméti, Á., **Nagy, C.**, Csősz, É., Kalló, G., Gáspár, A.: The application of a microfluidic reactor including spontaneously adsorbed trypsin for rapid protein digestion of human tear samples.
Proteom. Clin. Appl. 11 (11-12), 1-9, 2017. ISSN: 1862-8346.
DOI: http://dx.doi.org/10.1002/prca.201700055
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