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The advantage of mass spectrometry in the electrophoretic analysis of acidic and amphoteric small and macromolecules in aqueous and non-aqueous systems

PhD thesis

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I. Introduction

Capillary electrophoresis (CE) is a modern, powerful analytical technique used for the separation of both positively charged, negatively charged and uncharged compounds even in the same measurement. Although the method was first used by Tiselius in 1937, the basis of CE was laid down in 1967 by Hjertén who first showed the possibility (theory, advantages and disadvantages) of separating various compounds using rotating tubes of 3 mm inner diameter in absence of conventional supports in free zone electrophoresis. During the years, different CE modes have been developed and studied in CE, namely capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), isotachophoresis (ITP), capillary isoelectric focusing (CIEF) and capillary electrochromatography (CEC). CE has now become a commonly used separation technique since it requires a very small amount of sample (1-10 nL), easy to automate and set up, has a short analysis time, and extremely versatile.

Several detection strategies are used in CE. In the past, most commonly ultraviolet or fluorescence detectors were used after removing the external coating on a small part of the capillary layer. Other instruments were equipped by conductivity, fluorescence, amperometric or laser induced fluorescence electrochemical devices as detectors. Nowadays, mass spectrometry (MS) is the most commonly used detection in CE. The coupling of CE with MS results in a very powerful analytical tool since it combines the outstanding detection sensitivity, the ability of providing structural information and the high separation efficiency of CE. Thus, CE-MS offers both the detection and the structure determination of the unknown analyte.

Capillary electrophoresis is able to avoid the use of organic solvents for separation. Aside from environmental and toxicity concerns, Non-aqueous Capillary Electrophoresis (NACE) is considered when the compound is not soluble in common aqueous solvents, buffers and surfactants, such as hydrophobic ionic compounds. In addition, most organic solvents are more volatile and have lower surface tension than water, making NACE more suitable for online coupling with MS. As a result, NACE-MS becomes very powerful and versatile,

as this analytical technique possesses many excellences: it is of high speed, is very efficient, and has a low sample consumption and high selectivity.

The developed methods presented in the thesis contribute to:

- the detection of six organic acids from wine samples and thus for quality control and origin testing of wines;

- establish a mass spectrometric hyphenation during capillary isoelectric focusing implemented with a sequential injection protocol, opening up new possibilities for the separation of proteins;

- the structural analysis of lipid A, determination of their phosphorylation and acylation profiles, thus for a more precise understanding of bacterial infections and their induced immune responses.

II. Aims of the study

During our work, we focused on the development of robust and reliable methods that enable the coupling of capillary electrophoresis and mass spectrometry in both aqueous and non-aqueous systems. The developed aqueous and non-aqueous methods were applied to the analysis of biologically important samples.

Our main goals:

• Determination and quantification of organic acids in red wines using the CZE-ESI/QTOF-MS method optimization.

• Development of a sequential injection protocol for efficient introduction of protein samples into the capillary for mass spectrometric analysis, which avoids the interfering effect of ampholytes.

• Separation and characterization of lipid A isomers based on their phosphorylation and acylation patterns using the CE-MS method in a non-aqueous system (eliminating solubility limits).

• Selection of appropriate CE polarities and MS detection conditions, including the application of suction and external pressure, in order to identify lipid A molecules through fragmentation patterns in negative and positive ionization modes.

• Separation and characterization of the phosphorylation and acylation isomers of lipid A using CE-MS and CE-MS/MS methods.

III. Materials and methods

1. Chemicals

Chemicals used during Macedonian Vranec wine analysis - lactic, succinic, malic, tartaric, shikimic and citric acid standards, polybrene (hexadimethrine bromide, PB), acetic acid, formic acid, ammonium hydroxide and sodium hydroxide - were supplied from Sigma-Aldrich (Steinheim, Germany). Ultra-pure deionized water (LC-MS Chromasolv®) was obtained from Fluka (Buchs, Switzerland).
For protein separations, water, methanol (LC–MS CHROMASOLV quality), formic acid (eluent component in LC–MS) and ammonium hydroxide solution (28.0–30.0%), lysozyme (14.3 kDa) from chicken egg white (≥90%), cytochrome c (12.4 Da) from equine heart (≥95%), myoglobin (17.0 kDa) from equine heart (≥90%), and β-lactoglobulin A (18.4 kDa) from bovine milk were purchased from Sigma–Aldrich (Steinheim, Germany, and Saint Louis, MO, USA). Phosphoric acid (85 m/m %), sodium hydroxide, and hydrochloric acid were obtained from Spektrum 3D (Debrecen, Hungary).
The Lipid-A analysis was performed by using methanol (MeOH) (LC-MS Chromasolv®), dichloromethane (DCM) (Chromasolv Plus, for HPLC, ≥99.9%), triethylamine (TEA) and acetic acid (AcOH) (eluent additives for LC-MS), sodium hydroxide (NaOH), and acetonitrile (ACN) purchased from Sigma-Aldrich (Steinheim, Germany).

2. Applied instruments and software

During our work, we worked with an Agilent 7100 capillary electrophoresis instrument and an Agilent 6530 Q-TOF LC-MS system. The two devices were connected via an Agilent Jet Stream ESI interface. For control of the CE device and data processing, ChemStation B. 04.03. and 7.01 were used. The mass spectrometer was controlled by Agilent MassHunter B.04.00 software.

3. Measurement conditions

In case of the wine sample, an 80 cm long, 50 µm internal diameter capillary covered with polybrene was used. -20 kV was the applied

voltage difference. The sheath liquid (1% v/v solution of formic acid) was delivered at 0.7 µL/min flow using an LC isocratic pump (1260 Infinity series, Agilent Technologies, Waldbronn, Germany). The ESI/QTOF-MS was operated in the negative ionization mode applying electrospray voltage of 4.5 kV. Nitrogen was used as drying gas at 325°C, with a flow rate of 8 L/min; the pressure of the nebulizer gas was set at 35 psi. The sheath gas temperature was 350°C, with flow rate of 11 L/min. The TOF-MS parameters were the following: fragmentator, 100 V, and skimmer, 65 V. The scanning mass-to-charge (m/z) range of the time-of-flight analyzer was 50–250 m/z with a maximum accumulation time of 1000 ms/spectrum.

During isoelectric focusing of proteins, Ampholine (pH 3.5–10; pH 7–9; pH 4–6) ampholytes were used. Experimental conditions: PAA-coated capillary 70 cm (61.5 cm to UV detector) × 50 µm inner diameter; anolyte 50 mM formic acid, pH 2.3; catholyte, 100 mM ammonium hydroxide, titrated to pH 8.5 with 50 mM formic acid. Sequential injection of ampholyte/sample/ampholyte zones at 50 mbar were the following: "40/6/40"; "80/6/80"; "160/6/0"; "0/6/160"; "40/6/0" or “0/6/40”. The applied voltage difference was +20 kV (field strength 286 V/cm); mobilization: after 10 minutes of focusing with 50 mbar pressure (the voltage difference maintained). The Q-TOF MS instrument was operated with an Agilent Jet Stream ESI ion source in positive ionization mode with a mass accuracy of <10 ppm, a mass resolution of 10000 –20000 (121–2722 m/z), a measuring frequency of 10000 transients/s and a detection frequency of 2 GHz (200000 points/transient). The Jet Stream ESI ion source was operated using the following conditions: pressure of the nebulizing gas (N_2) was 20 psi, the temperature and flow rate of the drying gas (N_2) were 300°C and 7 L/min, and the temperature and flow rate of the sheath gas (N_2) were 300°C and 11 L/min, respectively. The ESI capillary voltage was 4000 V, fragmentor and skimmer potential were set to 200 and 65 V, respectively. The sheath liquid (4:1 mixture of methanol/water containing 1% v/v formic acid) was delivered at a flow rate of 10 µL/min.

For Lipid A samples, the composition of the optimal background electrolyte solution was MeOH:DCM:TEA:AcOH (40:60:1.08:0.36,

v/v/v/v). The measurements were carried out in untreated quartz capillaries (50 µm inner diameter and 55 cm length) using a voltage difference of +30 and -30 kV. The original Agilent stainless steel ESI needle was replaced with an Agilent G7100–60041 platinum needle. MS conditions: sheath fluid contained 0.06% (v/v) TEA and 0.02% (v/v) AcOH in MeOH at a flow rate of 5 µL/min delivered by an isocratic pump (Agilent Technologies) through the splitter at a ratio of 1:100. The electrospray voltage was set at 3.0 kV in both negative and positive ion modes. The nitrogen drying gas flow rate was 5.0 L/min at 200°C and 15 psi. Identification of ion peaks was achieved by CID MS/MS experiments in both positive- and negative-ion modes by applying precursor m/z-dependent linear collision energy gradient. The slope of the linear energy gradient was 0.026 in the positive- and 0.044 in the negative-ion mode, and the MS/MS spectra were acquired with auto MS/MS mode in the range of m/z 50–2100 at the scan rate of 2 spectra/s.

IV. Results

1. Results of method developments in aqueous system

i. CZE-ESI-QTOF-MS analysis of Vranec wine sample from Macedonia

A rapid separation method was developed using CZE-ESI/QTOF-MS, with which it was possible to determine the concentrations of different organic acids from a Macedonian red wine sample. Organic acids separated in wine: lactic acid, succinic acid, malic acid, tartaric acid, shikimic acid and citric acid.

During the experiments, two volatile buffers, ammonium acetate and ammonium formate, were tested in order to optimize MS detection. Baseline separation of individual compounds was not achieved with either background electrolyte, however this was not necessary as adequate extracted ion electropherograms were produced and partial separation still provided benefits. Ammonium acetate resulted in excellent extracted ion peaks, so it was selected as the background electrolyte. Increasing the concentration of ammonium acetate from 10 to 75 mM led to peak broadening, while lower background electrolyte concentrations resulted in longer migration times. The 50 mM ammonium acetate buffer (pH=6.0) was chosen as the optimal condition for the separation of organic acids.

Neither the 80 cm nor the 120 cm capillary was used to achieve a baseline separation of the individual compounds. It should be noted that tartaric acid, malic acid and succinic acid migrated close to each other, followed by lactic acid, shikimic acid and citric acid. Due to the 4-minute run time, an 80 cm capillary was selected for the experiments.

In order to maintain separation and peak shape, a potential difference higher than 20 kV was not used, as increasing the voltage resulted in worse resolution and peak broadening. During the experiments, 1% v/v formic acid was used as a sheath liquid, which adequately promoted the ionization of organic acids.

ii. CIEF-sequential injection-ESI-QTOF-MS analysis of protein samples

In the course of method development, a protein mixture comprising four proteins (lysozyme with a pI of 11.35, cytochrome c with a pI of 10.25, myoglobin with a pI of 6.8, and β-lactoglobulin A with a pI of 5.3) was applied as the sample. Using a sequential injection protocol, both ampholyte and sample mixture were introduced into a 70 cm long PAA-coated capillary in CZE-ESI/QTOF-MS analysis. Monitoring was performed through UV and MS detection during CIEF measurements. The isoelectric pattern's mobilization to the UV detector involved applying 50 mbar pressure after a 10-minute focusing step, with the maintenance of the focusing voltage. A noteworthy observation was made during the use of a 1% Ampholine pH 3.5–10 ampholyte solution in a "40/6/40" injection protocol, as compared to the experiment with half (0.5% v/v) ampholyte concentration but a longer "80/6/80" injection protocol. Comparisons between different injection protocols, such as "80/6/80", "160/6/0", or "0/6/160", were conducted using 0.5% Ampholine pH 7–9. It can be observed that the length of the ampholyte zones remained almost the same. It is particularly noteworthy that when the ampholyte with a wide pH range was used, the two basic proteins could not be well separated, although the other two proteins were separated at the baseline level. However, when ampholytes with a narrow pH range (pH 7–9) were used, under similar experimental conditions (applied pressure, mobilization), the two basic proteins that were not separated migrated to the cathode side of the pH gradient.

The separation of the four proteins was performed using Ampholine pH 7–9 ampholytes with MS detection after introducing significant modifications in the CIEF experiments. Even though the PAA coating does not mobilize the pH gradient, the coupling of the CE instrument to the MS via the ESI interface induces zone mobilization (nebulizer gas suction effect). Even in the absence of pressure, the components reached the MS detector significantly faster than in the previous experiment using UV detection. The application of a "40/6/40" injection protocol (utilizing only 0.5 v/v% ampholytes) resulted in the

separation of the four proteins, although with varying resolution. In the case of half sandwich injection, the ampholytes were applied only after the injection of the sample components, as indicated by the "0." The use of half the amount of ampholytes, "0/6/40," led to a halved gradient zone length.
The effect of the ampholyte concentration on the separation efficiency and the pH gradient was observed for three different concentrations with Ampholine pH 4-6 solution. Decreasing the ampholyte concentration from 1% to 0.5% and 0.25% results in sharper protein peaks and shorter pH gradient zones. Although the bands of the two basic proteins overlap (they are indistinguishable from each other), myoglobin and β-lactoglobulin A are well separated at the baseline.

2. Results of method developments in non-aqueous system

i. Lipid A NACE–ESI-QTOF MS/MS study

The initial experiments aimed to achieve the separation of nonderivatized bacterial phosphoglycolipids by NACE–ESI-MS. Given the inherent heterogeneity of lipid A, various species were concurrently present in the sample, discerned through high-resolution Q-TOF MS measurements. The primary separation solvent for analyzing lipid A anions from S. sonnei consisted of pure methanol with the addition of 0.36:0.12 (v/v) TEA:AcOH.

ii. Separation efficiency and selectivity

In order to optimize the efficiency and selectivity of the separation, the effect of the solvent composition, electrolyte composition and electrolyte concentration of the background electrolyte solution was investigated, with the aim of what it causes in the migration time, separation efficiency and resolution.

iii. Determination of phosphorylation site and acylation profile by mass spectrometry

The enhanced solubility of lipid A molecules in the BGE containing 40–60% DCM resulted in increased intensity of the detected MS signals and lower detection limits. This improvement facilitated the application of MS/MS fragmentation measurements for accurate structural identification. TEA was applied as an additive to both the BGE and sheath liquid, allowing efficient ionization of lipid A components in both negative- and positive-ion modes (in two separate runs) as deprotonated molecules $[M - H]^-$ or as triethylammonium adduct ions $[M + H + TEA]^+$, respectively.

In the positive ion mode tandem mass spectra, dominant B-type ions (B_1 and B_2, which were formed by the cleavage of the glucosamine disaccharide glycosidic bonds in the C1' and C1 positions) were observed. These fragmentation ions serve as diagnostic ions to detect the phosphorylation pattern of lipid A. For example, the B_2 ion indicates the phosphorylation state at the C1 position by the loss of the added adduct forming agent (TEA) and the C1 substituent (ie, H_3PO_4 for C1-phosphorylation or H_2O for C4'-phosphorylation). Furthermore, the B_1 ion provides indirect information about the phosphorylation state at the C4' position.

In the positive ion mode tandem mass spectra, two different B_1 ion peaks appeared next to the same B_2 ion peak at 1264 m/z, which provides evidence for the presence of two tetra-acylated C4'-monophosphorylated lipid A isomers (compounds P4' and P4'*) with different acylation patterns.

Tandem mass spectra recorded in negative-ionization mode provide valuable information about lipid A acylation patterns. During the $[M–H]^-$ fragmentation of deprotonated monophosphorylated lipid A precursors, ester-linked fatty acids (both primary and secondary) are cleaved. Due to the position of the single phosphate group that regulates the fragmentation processes, fatty acid losses can occur sequentially (in the case of C4'-phosphorylation) or competitively (in the case of C1-phosphorylation). Despite differences in the preference of ester-bond cleavages between phosphorylation positional isomer structures, highly similar tandem mass spectra are

generated, showing several product ions at the same m/z but with different relative intensities. The NACE–ESI-MS/MS method offers high selectivity and improved sensitivity, allowing for the exact determination and comparison of CID fragmentation processes of phosphorylation isomers.

V. Thesis points

1. We succeeded in developing robust and reliable methods by coupling capillary electrophoresis and mass spectrometry, both in aqueous and non-aqueous systems.
2. We managed to identify six different organic acids (lactic acid, succinic acid, malic acid, tartaric acid, shikimic acid and citric acid) and we determined the concentrations of these organic acids from a red wine sample using CZE-ESI/QTOF-MS.
3. The separation of proteins (lysozyme, cytochrome c, myoglobin, β-lactoglobulin A) was also carried out in an aqueous system using a sequential injection protocol with isoelectric focusing and MS detection. During our experiments, we changed the types of ampholytes with different pH ranges and their concentrations, "sandwich" and "half-sandwich" injection types, as well as the length of the injected zones.
4. We managed to separate the lipid A components using the CE-MS method in a non-aqueous system. During the measurements, four peak groups were distinguished during the 25 minute separation time, which represented non-, C4'-monophosphorylated, C1-monophosphorylated and bisphosphorylated lipid A. The solvent composition and concentration of the background electrolyte solution were changed in order to determine the effect on the migration time, separation efficiency and resolution.
5. Lipid A molecules were identified through fragmentation patterns in positive and negative ionization modes based on their phosphorylation and acylation patterns. Gradual exchange of methanol with DCM had a significant effect on the separation of critical pairs of tetra-acylation isomers. We also studied the change in the molar and volume ratios of TEA and AcOH electrolyte additives, both normal and in reverse CE polarity mode.

VI. Publications

1. Publications related to the thesis

- Violeta Ivanova-Petropulos, Zaneta Naceva, Viktor Sándor, Lilla Makszin, Laura Nagy, **Balázs Berkics**, Trajce Stafilov, Ferenc Kilár, Fast determination of lactic, succinic, malic, tartaric, shikimic and citric acids in red Vranec wines by CZE-ESI-QTOF-MS, ELECTROPHORESIS, 2018, 39(13), pp. 1597-1605 https://doi.org/10.1002/elps.201700492
- Csilla Páger, Nikoleta Biherczová, Roland Ligetvári, **Balázs Viktor Berkics**, Tamás Pongrácz, Viktor Sándor, Anita Bufa, Viktória Poór, Andrea Vojs Staňová, Ferenc Kilár, Advanced online mass spectrometry detection of proteins separated by capillary isoelectric focusing after sequential injection, JOURNAL OF SEPARATION SCIENCE, 2017, 40(24), pp. 4825-4834, https://doi.org/10.1002/jssc.201700695
- Viktor Sándor*, **Balázs Viktor Berkics***, Anikó Kilár, Béla Kocsis, Ferenc Kilár, Ágnes Dörnyei, NACE–ESI-MS/MS method for separation and characterization of phosphorylation and acylation isomers of lipid A, ELECTROPHORESIS, 2020, 41(13-14), pp. 1178-1188, https://doi.org/10.1002/elps.201900251

*** Shared first authorship with Dr. Viktor Sándor**

2. Non-referred conference abstracts related to the thesis

- **Berkics Balázs Viktor**; Pongrácz Tamás; Poór Viktória; Sándor Viktor; Csóka Balázs; Fenyvesiné Páger Csilla; Kilár Ferenc, Nitrofenol festékek és fehérjék elválasztása kapilláris izoelektromos fókuszálással, izoelektromos fókuszálás kapcsolása tömegspektrométerrel, Elválasztástudományi

Vándorgyűlés 2014, Egerszalók, Magyarország 2014.10.12. - 2014.10.14.

- Fenyvesiné Páger Csilla; Biherczova Nikoleta ; Ligetvári Roland; **Berkics Balázs Viktor**; Sándor Viktor; Kilár Ferenc, Kapilláris izoelektromos fókuszálás alkalmazása tömegspektrometriás detektálással fehérjekeverék elválasztására, Elválasztástudományi Vándorgyűlés 2016, Kecskemét, Magyarország 2016.11.09. - 2016.11.11.
- Violeta Ivanova - Petropulos ; Zaneta Naceva ; Viktor Sándor; **Balázs Berkics**; Trajce Stafilov ; Ferenc Kilár, Determination of Organic Acids in Wines Using Capillary Zone Electrophoresis - Electrospray Ionization / Quadrupole - Time - of - Flight - Mass Spectrometry (CZE - ESI/QTOF - MS), 16th CEEPUS Symposium and Summer School on Bioanalysis, Warsaw, Lengyelország 2016.07.06. - 2016.07.15.
- Sándor Viktor; Kilár Anikó; **Berkics Balázs**; Kocsis Béla; Kilár Ferenc;; Dörnyei Ágnes, Comparison of LC-MS and CE MS for the analysis of immunstimulant bacterial membrane glycolipids, 12th Balaton Symposium on High-Performance Separation Methods, Siófok, Magyarország 2019.09.11. - 2019.09.13.