HILIC: EFFECT OF THE STRUCTURE OF THE MOBILE AND STATIONARY PHASE ON THE RETENTION OF PHENOLIC COMPOUNDS ON THE DIOL BASED COLUMNS

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1. Introduction

1.1. HILIC

HILIC, which is a useful method for separation of polar compounds\(^1\), can be characterized as a normal-phase liquid chromatography using the polar columns and water-organic mobile phases, where the range of content of water component is around 2–40 % (ref.\(^2\)). The principle of the method consists in adsorption of molecules water from the water-organic mobile phase onto the surface of the polar stationary phase, where it creates a diffuse layer. The retention is based on the combination of two major mechanisms, partition into the water-rich layer and adsorption onto the surface of a polar stationary phase\(^3\).

1.2. Abraham LSER model

LSER model is the approach, which employs multi-parameter linear correlation between the solvation parameters and the logarithm of the solute retention factor\(^4\) where V,S,A,B are the structural molecular descriptors and v,s,a,b are the separation system parameters.

2. Experimental

The experiments were carried out on the YMC Triart Column (5 μm, 150 × 2 mm id, organic/inorganic silica particles with bonded dihydroxypropyle groups) and the results were compared to the other two columns with the diol-based columns, Luna HILIC (3 μm, 50 × 3 mm id) and LiChrospher 100 Diol (5 μm, 125 × 4 mm id). All the experiments were carried out at 40 °C at the flow-rate of the mobile phase set to 0.2 mL min\(^{-1}\). The composition of the mobile phase was: 10 mM NH\(_4\)Ac in water with the addition of 0,1 % HCOOH/10 mM NH\(_4\)Ac in acetonitrile. The standard compounds were phenolic acids and flavonoids.

3. Results and discussion

3.1. Effect of the mobile phase on the retention of flavonoids and phenolic acids

The retention was measured over the full mobile phase composition range—from 2 to 95 percent of buffered water (10 mM NH\(_4\)Ac, 0,1% HCOOH) in acetonitrile. The columns showed both reversed phase and aqueous normal phase (HILIC) mechanism and this dual retention mechanism was successfully described by the four parameter equation\(^5\):

\[
\log k = a + m_{\text{RP}} \cdot \phi_{\text{H2O}} - m_{\text{HILIC}} \cdot \log(1 + b \cdot \phi_{\text{H2O}})
\]

where a is the logarithm of the retention factor in pure less polar solvent. Parameters \(m_{\text{HILIC}}/m_{\text{RP}}\) characterize the rate of decreasing/increasing retention with increasing volume fraction of buffered water in the mobile phase. All plots of retention factor (k) dependence on the content of water showed "U-shape" (ref.\(^5,6\)).

3.2. Effect of the structure of the mobile and stationary phases on the retention of phenolic acids and flavonoids – Abraham LSER model

The parameters v, s, a, b (\(\nu\)“ characterize the contribution selective non-polar, “s” dipole–dipole and “a

![Fig. 1. Dependence of the logarithm of the retention factor of \(\rho\)-hydroxybenzoic acid on the volume fraction of water on the YMC Triart Diol-HILIC column. The volume fraction of water, \(\phi_{\text{H2O}}\), corresponding to the minimum of the U-turn, indicates the transition between the HILIC and RP modes, and \(\phi_{\text{a}}\) correspond to the volume fraction of water, where the retention of the phenolic acids and flavonoids in the HILIC mode is sufficient. Values of \(\phi_{\text{a}}\) and \(\phi_{\text{a}}\) were compared to the other columns and widest range of composition of the mobile phase, where HILIC mechanism predominates, was on the YMC Triart Diol HILIC column.](image-url)
and b" hydrogen-bonding interactions) were obtained as a result of multilinear regression analysis fitting the experimental retention factors, k, to the LFER model described by eq. (1) and are shown in Table II. Fitting the experimental data with Abraham LFER model was the most successful for flavonoids in the RP mode. For phenolic acids was the experimental fitting as similar in HILIC as in RP mode. The retention of phenolic acids and flavonoids is most affected by the parameter v, which characterizes contribution of the size of the molecule (dispersion interactions) to the retention and b, which characterizes contribution of the hydrogen bonding basicity.

3.3. Comparison of the separation properties of the columns

The separation selectivity was investigated by calculating the separation factors, α, which signify the relation of the retention factors of phenolic acids to the retention factor of 4-hydroxyphenylacetic acid. The separation factors of phenolic acids were the highest in the HILIC mode on YMC-Triart Diol-HILIC (Table III).

4. Conclusions

As a result of the comparison, Ymc Triart Diol HILIC showed better separation selectivity for phenolic acids, higher relative retention in the HILIC mode and wider range of volume fraction of buffered water in the mobile phase, where the HILIC mechanism predominates. The column has acidic properties and the contribution of dipole-dipole interactions is apparent in HILIC mode. The hydrophobic interactions are lesser in RP mode in comparison to other columns.

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REFERENCES

### Table III
Separation factors for phenolic acids in the RP (95% H₂O) and HILIC (2% H₂O) modes

<table>
<thead>
<tr>
<th>P. acids</th>
<th>YMC Triol-Diol-HILIC</th>
<th></th>
<th>LiChrospher DIOL</th>
<th></th>
<th>Luna HILIC</th>
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<tr>
<td></td>
<td>k&lt;sub&gt; RP&lt;/sub&gt;</td>
<td>α</td>
<td>k&lt;sub&gt; HILIC&lt;/sub&gt;</td>
<td>α</td>
<td>k&lt;sub&gt; RP&lt;/sub&gt;</td>
<td>α</td>
</tr>
<tr>
<td>1 Sal</td>
<td>0.45</td>
<td>1.52</td>
<td>2.75</td>
<td>3.08</td>
<td>0.53</td>
<td>3.55</td>
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<tr>
<td>2 Cou</td>
<td>0.62</td>
<td>2.07</td>
<td>7.02</td>
<td>1.21</td>
<td>0.51</td>
<td>3.45</td>
</tr>
<tr>
<td>3 Phb</td>
<td>0.39</td>
<td>1.32</td>
<td>6.34</td>
<td>1.33</td>
<td>0.32</td>
<td>2.16</td>
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<tr>
<td>4 Fer</td>
<td>0.74</td>
<td>2.50</td>
<td>5.89</td>
<td>1.44</td>
<td>0.61</td>
<td>4.10</td>
</tr>
<tr>
<td>5 Van</td>
<td>0.48</td>
<td>1.60</td>
<td>6.57</td>
<td>1.29</td>
<td>0.37</td>
<td>2.53</td>
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<tr>
<td>6 Sin</td>
<td>0.92</td>
<td>3.07</td>
<td>6.07</td>
<td>1.39</td>
<td>0.66</td>
<td>4.48</td>
</tr>
<tr>
<td>7 Syr</td>
<td>0.34</td>
<td>1.81</td>
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<td>0.99</td>
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<td>2.65</td>
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<tr>
<td>8 Hpa</td>
<td>0.30</td>
<td>1.00</td>
<td>8.46</td>
<td>1.00</td>
<td>0.15</td>
<td>1.00</td>
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<tr>
<td>9 Gal</td>
<td>0.26</td>
<td>1.16</td>
<td>/</td>
<td>/</td>
<td>0.19</td>
<td>1.28</td>
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<tr>
<td>10 Chl</td>
<td>0.19</td>
<td>1.54</td>
<td>/</td>
<td>/</td>
<td>0.09</td>
<td>1.68</td>
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MULTI-STEP SYNTHESIS OF CASPASE-3 SENSOR BASED ON FÖRSTER RESONANCE ENERGY TRANSFER

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Summary

Programmed cell death or apoptosis is regulated process of cell suicide. The central role in apoptosis play cysteine proteases called caspases. Caspases recognize tetra-peptide sequences Asp-Glu-Val-Asp (DEVD) on their substrates and hydrolyze peptide bonds after aspartic acid residues. Various techniques for the determination of caspase-3 are commercially available e.g. Enzyme Linked Immuno-Sorbent Assay (ELISA), Western blotting or flow cytometric analysis. The products of the cleavage can be detected by spectrophotometry, fluorimetry, chemiluminescence (CL) or ELISA. In this work, we suggested fluorescent sensor based on Förster Resonance Energy Transfer (FRET) to determine caspase-3 in cell nucleus or cytoplasm by apoptosis for very fast analysis by fluorescence microscopy without sample destroying.

1. Introduction

Programmed cell death (apoptosis) is an essential mechanism to eliminate unwanted cells during the development and homeostasis of multicellular organism1-2. Unregulated cell death is implicated in a growing number of clinical disorders. Failure of apoptosis in different way can lead to ischemia damage, neurodegenerative diseases, cancer or autoimmune diseases3,4. The central component of apoptosis is a proteolytic system involving a family of cysteine proteases called caspases. Caspases are among the most specific proteases, an unusual and absolute requirement for cleavage of the peptide bond after aspartic acid (C-terminal)5,6. Caspase family consists of 15 mammalian members. Caspases are potential targets for therapeutic interventions in the case of tumorigenesis and other pathologies7,8. One of the members of caspase family caspase-3 is a key factor in apoptosis which recognizes tetra-peptide sequences DEVD. Currently, the majority of analytical methods for detecting caspase-3 activity are Western blotting, flow cytometric analysis or ELISA with colorimetric/fluorimetric detection9,10. Plenty of techniques were recently developed for detection of caspase-3 in cells with high spatio-temporal resolution e.g. fluorescence resonance energy transfer (FRET) based assay11,12. Förster (fluorescence) resonance energy transfer (FRET) is a widely prevalent photophysical process that occurs between a donor (D) molecule in the excited state and an acceptor (A) molecule in ground states. Energy transfer occurs without the appearance of a photon and is the result of long range dipole-dipole interactions between D and A. This physical transfer of energy usually takes place over a D-A separation of 0.5–10 nm (ref11-13). FRET is one of the few experimental techniques that are able to detect and define distance between molecules, molecular dimensions, proximities change with time, heterogeneous molecular conformations etc.14.

Luminescent semiconductor nanocrystals called quantum dots (QDs) have unique photophysical properties e.g. high photostability, high emission quantum yield, narrow emission peaks and size – dependent wavelength tunability. QDs have already been used successfully in cellular imaging, immunoassay, DNA hybridization and optical barcoding. QDs with high photostability and wavelength tunability are very suitable to use in FRET based assay as a donor of transferred energy14.

2. Experimental

For QDs preparation was used one-step synthesis described elsewhere15. For organic quencher preparation was used synthesis described in US patent16.

2.1. Chemicals

As a linker chain were used cysteamine (98%) and O-(3-Carboxypropyl)-O’-[2-(3-mercaptopropionylamino)ethyl]-polyethylene glycol (PEG) (Mw 3000), as a DEVD sequence was used Enzyme Linked Immuno-Sorbent Assay (ELISA), Western blotting or flow cytometric analysis. The products of the cleavage can be detected by spectrophotometry, fluorimetry, chemiluminescence (CL) or ELISA. In this work, we suggested fluorescent sensor based on Förster Resonance Energy Transfer (FRET) to determine caspase-3 in cell nucleus or cytoplasm by apoptosis for very fast analysis by fluorescence microscopy without sample destroying.
Neptune Purite Ultimate. For TLC detection we used TLC Silica gel 60 (Merck) and mobile phase was methanol (for UV-VIS spectroscopy) from PENTA.

2.2. Conjugation reactions of QDs and linkers

At first, QDs were conjugated with cysteamine as a linker. QDs (5 mg) were dissolved in 1 ml of 50 mM carbonate buffer at pH 10.5. Then 5 mg EDC and 2 mg sulfo-NHS were add and gently vortexed. This mixture then reacted with 15 mg cysteamine to produce conjugate with terminal thiol group for subsequent reactions. This conjugate was precipitate using i-Pr and dried in vacuum in Concentrator 5301 (Eppendorf) at 30 °C. Dried conjugate of QDs and cysteamine was dissolved in 0.5 mL carbonate buffer (pH 10.5). This solution then reacted with 78 μl of PEG (1 mg mL⁻¹ in 50 mM carbonate buffer, pH 10.5). Reaction mixture reacted for 2 hours at 37 °C to produce disulfide bridges. Reaction product was precipitate using i-Pr and dried in vacuum in Concentrator 5301 at 30 °C.

2.3. Reduction and purification of DEVD sequence

Nitro group from p-nitroanilide in DEVD sequence was moderately reduced using sodium dithionite. 2.5 mg DEVD was dissolved in deionized water with addition of 6 μl sodium hydroxide (c = 1.25 M) in order to increase DEVD solubility. 11 mg of Na₂S₂O₄ was then added into alkalized solution of DEVD and reduction reaction occurred immediately. Successful reduction of nitro group in DEVD sequence was checked by TLC at 254 nm and by ninhydrin reaction. As a mobile phase was used methanol.

Desalting of reduced DEVD was done by Zip-Tip C18 from Millipore.

3. Results and discussion

We have designed sensor for caspase-3 determination in individual apoptotic cells. This sensor is based on FRET, were oscillating electrons, in donor in our case quantum dot, exchange energy with acceptor dipole (BHQ-2 modified quencher) with similar resonance energy via the chain of linker. Our modified BHQ-2 quencher absorbs transported energy and is able to quenched fluorescence. But after cleavage of the bond between quencher and DEVD sequence in presence of caspase-3 in real samples, there is no non-radiative FRET transfer, quencher doesn’t absorb energy and QD emits light at given wavelength (Fig. 1). We used CdTe QDs with maximum emission wavelength 600 nm and with mercaptopropionic acid as a surface ligand.

QDs were modified for DEVD bonding at first by cysteamine (as short linker) and at second by PEG to achieve efficient and optimal Förster distance. Products analyses of conjugation reactions were checked by capillary zone electrophoresis with laser induced fluorescence detection (CZE-LIF). Quencher was successfully synthesized according US patent¹⁶. We succeed in solving problems with QDs precipitation during sensor synthesis and problems with purification of particular conjugation products. We successfully desalted reduced DEVD and checked it by MALDI-TOF. All results will be presented.

![Fig. 1. Model of caspase-3 sensor with DEVD sequence. A) Model of caspase-3 sensor, without cleavage of DEVD chain and FRET transfer with quenching of fluorescence, B) Cleavage of bond between DEVD and quencher and no FRET transfer with QD’s emission of light](image-url)
4. Conclusions

We designed new sensor based on FRET to determined caspase-3 in individual apoptotic cells especially to determine caspase-3 in cell nucleus or cytoplasm. Using of this sensor offer us possibility for very fast analysis of samples by fluorescence microscopy. This method allow reusing of samples in comparison with other detection techniques of caspase-3 like ELISA where samples are destroyed e.g. by cell lysis.

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REFERENCES

CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION FOR AMINO ACID PROFILING OF HUMAN AND URINE SAMPLES

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Summary

Analysis of amino acids in their native form using conventional detection techniques is hindered by lack of chromophores or fluorophores in the structure of the most amino acid. As a result, amino acids are prior the detection derivatized by suitable agents. Derivatization step can be omitted when conductivity detection method is employed. Benefits of capillary electrophoresis and contactless conductivity detection were merged and resulted in analytical method that was tested for amino acid profiling of human plasma and urine samples.

1. Introduction

Quantitative amino acid (AA) analysis is a significant tool for many scientific areas ranging from the characterization of protein to the description of natural products attractive for food and pharmaceutical industry. AAs are not only building blocks of proteins but they also act as precursors of several neurotransmitters, porphyrins and nucleic acids. Quantification of AAs in biological fluids can reveal metabolic disorders affecting synthesis or degradation of AAs. Since AAs play complex role in a living organism, they can reflect health-sate of the organism thus AA profiling can be used for supplementary diagnostic purposes in medical practice where determination of proper diagnosis is unclear or when specific markers are not discovered yet.

2. Experimental

Agilent G7100 Capillary Electrophoresis System with integrated A/D converter using in-cassette-built in-house assembled contactless conductivity detection (CE-C4D) was used for analysis of AAs in their native form. C4D was derived from the one presented by Gas et al. differing mainly in an operational frequency of a crystal oscillator used. Data acquisition and integration were performed by Agilent ChemStation software.

2.1. Separation conditions

Separation conditions follow from the articles published earlier and were refined to obtain the best performance for separation of human plasma, serum and urine samples. Background electrolyte (BGE) consists of 8% (v/v) acetic acid and 0.1% (m/m) (hydroxyethyl)-cellulose. Separations were carried out in a bare fused-silica capillary of 50/375 µm inner/outer diameter and 80.0/65.6 cm total/effective length. Cassette with capillary was kept at constant 30 °C and a driving voltage was set to +30 kV with a typical current 12 µA (anode at a sample introduction side). Sample was introduced into the capillary by 50 mbar (0.725 psi) pressure drop for 30, 24 and 12 seconds for human plasma, urine and for creatinine (CR) determination in urine, respectively.

2.2. Sample treatment

Samples of human plasma and urine were stored frozen at –70 °C. On the day of the analysis they were thawed at a room temperature. Proteins were precipitated by an addition of acetonitrile in the volume ratio 1:2, thoroughly stirred and centrifuged at 10 000×g for 10 minutes. Supernatants of human plasma samples were enriched by an addition of guanidineacetic acid (GAc; internal standard) giving final concentration of 50 µM of GAc, 3.1× diluted sample in the 66 % (v/v) acetonitrile. Supernatants of urine samples were analyzed without GAc due to co-migration of GAc with other unidentified peaks, thus the urine samples were only 3× diluted in 66 % (v/v) acetonitrile. Determination of CR in the urine samples demands additional dilution due to a high concentration of CR in the urine. Urine samples were diluted in 66 % (v/v) acetonitrile giving final dilution 100–200×. Presence of acetonitrile in the samples reduces sample conductivity and results in better peak shapes.

3. Results and discussion

3.1. Plasma samples

Twenty-six human plasma samples were collected from patients diagnosed with i) cancer, ii) cystic fibrosis, iii) coagulation defects, purpura and other hemorrhagic conditions, iv) other venous embolism and thrombosis. Samples were both from men and women on average 31 years old (range 1–66 years). Quantification of 18 AAs was possible in the all samples. Measured concentrations of AAs were submitted to principal component analysis using OriginPro software resulted in a partial grouping of the samples with the same diagnosis.
3.2. Urine samples

Four early urine samples were provided voluntarily by women 21–24 years old. Prior to storage, the urine samples were passed through membrane filter with 0.2 μm porosity to remove cells and concretions. One urine sample was provided by a woman suffering from epilepsy seizures (cause untold) and the rest were from healthy women and served as a control group. There were found significant differences in levels of the most quantified AAs. However, no solid conclusion can be stated because of a small statistical sample.

4. Conclusions

Presented CE-C4D method proved to be sensitive and reliable for analysis of AAs their native form in human plasma and urine samples. Multivariate statistical analysis resulted in a partial grouping of the human plasma samples with the same diagnosis thus AA profiling can provide supplementary information about the health-state of the organism. Urine analyses showed sensitivity and precision of the CE-C4D method. Fact that both plasma and urine can be analyzed at the same condition differing only in the sample introduction times benefits for easy automation of the method.

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ANALYSIS OF TISSUE-BOUND CELLS: NOVEL APPROACHES USING LASER CAPTURE MICRODISSECTION

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Summary

Recent biomedical research prefers analysis of homogenous samples obtained from primary cells to cell lines. Moreover, attention is paid to cells of low number but high biological impact, such as signalling centres in embryonic development, stem cells or tumour cells. To localize cells of interest within an organ/tissue, histological sections are widely used. However, the methods of choice for further analysis of such samples are mostly limited to histochemistry, immunohistochemistry and in situ hybridization.

The repertoire of methodical tools for specific analyses of tissue bound cell populations was considerably enriched by introduction of laser capture microdissection (LCM). The LCM system represents a modified microscopic technique and above other applications allows for selection of cells within histological sections and their visualisation of the section on a computer screen where the cells of interest can be gated. In the next step, laser beam contact-free laser-mediated catapult into a test-tube. This approach yields homogenous cell samples for further analyses. The basic principle operates with stained or unstained histological sections placed on a common or membrane coated slides. The LCM microscope allows visualisation of the section on a computer screen where the cells of interest can be gated. In the next step, laser beam cuts the area and catapults selected cells into a test tube.

Many significant results have been achieved using LCM technique during the last decade. However, as LCM usually provides small amount of biological materials, most of the results are related to the nucleic acid level where amplification methods are available. However, in the case of proteins, where the amplification step is not possible, the great benefits of LCM fade. Therefore, LCM applications in proteomics lack behind genomic and transcriptomic studies.

To overcome the disadvantage of low quantities of tissues and cells for protein evaluation, a novel approach based on flow cytometry (FC) of laser micro-dissected samples was designed. The technique combines LCM providing homogenous samples of tissue bound cells and flow cytometry allowing for evaluation of individual cells. To obtain intact cells, thick cryopreserved sections are used for LCM and the sample undergoes enzymatic and/or mechanical disintegration prior to FC. The method was verified using two populations of cells, apoptotic and proliferating, analysis was performed at DNA and protein levels. LCM-FC results were confronted with biochemical and immunohistochemical findings.

To go further, towards even more specific investigation, LCM has been considered with focus on single cells analysis and investigation of post-translational modifications of proteins. As a model, caspase-3 was used. This cystein protease represents one member of the cell death trio during apoptotic execution. Caspase-3 as an proenzyme is in general continuously expressed in cells and must be cleaved to become active. Three steps were followed in the development of novel precise methods. First, to evaluate the amount of active caspase-3 per cell in apoptotic vs. non-apoptotic cells, second to quantify caspase-3 in one single cell separated from a population, and last to detect caspase-3 in one cell obtained from the tissue using LCM.

To achieve the first goal, micromass cultures were challenged by camptothecin to stimulate apoptosis. Simultaneously, developmentally induced apoptosis was followed in digital and interdigital cell populations during mouse front limb digitalisation. A modified chemiluminiscence technique clearly showed quantitative differences in caspase-3 activation during naturally vs. experimentally induced apoptosis, increased amount of caspase-3 per apoptotic cells vs. non-apoptotic cells and was able to provide results with femtogram accuracy. To further precise the methods, micromanipulation system was used to select just one cell for analysis.

Recently, we have tackled the last step – to combine this novel precise technique and laser capture microdissection and thus design an elegant system for selection of one exact cell from the tissue and for analysis of this single cell. To make the method even more attractive, not only cryopreserved tissues but also fresh tissue slices have been tested. Tissue slices as designed a couple of years ago can be prepared using a tissue chopper and were successfully applied in several studies dealing with tracing of translocating cells within tissues.

Therefore, the novel methodical combination appears promising for basic research as well as several biomedical branches.

Recent research of tissue interactions is supported by the Grant Agency of the Czech Republic (P302/12/J059). Novel methodical approaches are granted by the project GACR P206/11/2377.
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DETERMINATION OF ORGANIC ACIDS IN COMPLEX SAMPLES BY USING UV AND MS/MS DETECTION WITH/WITHOUT HPLC SEPARATION

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Summary

The aim of this talk is to study different HPLC detectors for determination of both aliphatic and aromatic organic acids in complex mixtures. Due to the fact that suitable separation of these groups of compounds is requiring ion-exclusion chromatography (IEC) stationary phases, flow injection method was developed and tested for triple quadrupole MS detector.

1. Introduction

Separation, identification and quantitative analysis of aliphatic and aromatic acids are important due to their widespread presence in the environment and the use of both in medicine, agriculture and industry.

Free form aliphatic acids are found in various fruits e.g. malic acid in apples, citric acid in citrus fruits or tartaric, lactic and malic acids in grapes which concentration is necessary to know in winemaking. In the food industry are used as preservatives, for example benzoic acid as the sodium salt, which has the characteristics of an inhibitor for microorganisms. Carboxylic acids also serve as indicators of certain diseases, when their higher concentration e.g. in urine evokes a metabolic disorder called organic aciduria, which belongs to the hereditary metabolic disorders. The best known acidurias are propionic, glutaric, methylmalonic and pyroglutaric acid. Determination of carboxylic acids is very important for patients with diabetes, kidney disease and other metabolic disorders.

2. Experimental

2.1. Chemicals

All analytical standard-grade organic acids were obtained from Merck (Darmstadt, Germany) as the methanol (< 99% (v/v)) for liquid chromatography). Stock standard solutions were obtained by dissolution of the acids in Simplicity water or methanol. The Simplicity water was purified by passage through a Simplicity® Ultrapure Laboratory Water Systems (Molsheim – France). Potassium dihydrogen phosphate (KH2PO4) and 85 % (v/v) phosphoric acid (H3PO4) supplied by Merck (Darmstadt, Germany), 0.1 and 0.05% (v/v) formic acid (HCOOH) supplied by Merck (Darmstadt, Germany) were used for the preparation of mobile phase. Hydrochloric acid 37% (v/v) (Merck – Darmstadt, Germany) and ethyl acetate (Chemapol Group – Prague, Czech Republic) were used for human urine sample pretreatment.

2.2. Apparatus

Separation was carried out on a liquid chromatography Elite LaChrom (Merck – Hitachi, Darmstadt, Germany) equipped with pump L-2130, autosampler L-2200, thermostat L-2300, diode array detector L-2450, and Agilent 1290 UHPLC (Agilent Technologies, Waldbronn, Germany) equipped with binary pump, thermostated autosampler, thermostated column compartment, diode array detector, triple quadrupole MS/MS detector. Organic acids were separated on a silica based analytical column with specially modified reversed-phase functional group Alltech Preivall™ organic acid 5 μm (150 × 4.6 mm, I.D) with an Preivall organic acid 5 μm (7.5 × 4.6 mm, I.D) guard column (Grace – Deerfield, USA).

3. Results and discussion

The composition of the separation buffer solution and gradient experimental conditions have been optimized to achieve the best separation of organic acids. Aliphatic and aromatic acids were simultaneously separated using mobile phase composed of (A) 25 mmol L–1 KH2PO4 with pH 2.3 and (B) methanol with 25 mmol L –1 H3PO4 in ratio 80:20. The separation was performed with gradient elution at 25 °C and flow rate of 1 mL min–1. Injected volume of standard solutions mixture was 20 μL. The organic acids were detected with DAD at 220 nm.

The composition of mobile phase for MS detection was optimized using (A) 0.05% (v/v) formic acid and (B) methanol with 0.05% formic acid in ratio 80:20. Multi-mode ionization (ESI-APCI) in negative polarity mode was used for MS detection.

Flow injection analysis was performed restriction capillary (0.17 mm ID), and modified flow rate of mobile phase at 0.1 mL min–1. The composition of mobile phase for MS detection was optimized using (A) 0.05% (v/v) formic acid and (B) methanol with 0.05% formic acid in ratio 80:20. Multi-mode ionization (ESI-APCI) in negative polarity mode was used for MS detection.

The quantification of organic acids in samples was carried out by using the method of calibrations curve and standard addition method. We used the standard addition method to determine whether the matrix of a sample changes the analytical sensitivity.
4. Conclusions

The proposed HPLC method with DAD detection meets validation criteria for chromatography methods (ICH proposed criteria for validation of chromatography methods). MS/MS detection using triple quadrupole detector shows improvements of LOD, LOQ and provides valuable qualitative information further analysis including selectivity for coeluting compounds. Risk of false positive or negative identification and quantification is also improved using QQQ detection. Flow injection analysis with mass spectrometry detection shows perspectives in reducing analysis time significantly, further focus should be important for increasing sensitivity for biological fluids analysis. Work will be focused in the future on automated techniques for clinical laboratory purposes.

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Suppression of Protein Sample Losses in Autosampler Vials

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Summary

In the present study, we assessed peptide losses during sample storage in autosampler vials. We evaluated the possibility to suppress the adsorption effects by changing the composition of the sample solution injected into the liquid chromatography coupled with tandem mass spectrometry system (LC-MS/MS) and by use of different material of autosampler vials. As a model sample, a tryptic digest of six bovine proteins (B6E) in the amount of 1 fmol per protein was used for comparative experiments. The combination of a polypropylene vial and solution of poly(ethylene glycol) (PEG) (0.001%) or a mixture of highly concentrated urea and thiourea (5M and 1M) as injection solutions provided the best results in terms of number of significantly identified peptides \((P < 0.05)\). These conclusions were confirmed by analyses of a real sample with intermediate complexity. Addition of PEG into the real sample solution proved to prevent higher losses, concerning mainly hydrophobic peptides, during up to 48 h storage in the autosampler in comparison with a formic acid solution and even with a solution of highly concentrated urea and thiourea1.

2. Experimental

2.1. Vial materials

Autosampler vials from seven different materials were tested. We included 3 plastic-based and 4 glass-based commercially available autosampler vials: polypropylene (PP), polyethylene (PE), polymethylpentene (PMP), borosilicate glass (GL), KimshieldM glass (KIM), silanized glass (SIL), and RSA glass (RSA).

2.2. Sample injection solution

Effect of FA was compared with effects of other sample injection solutions: chaotropic agent urea (Urea), combination of urea with thiourea (U/T), dimethyl sulfoxide (DMSO), Anionic Acid Labile Surfactant I (AALS I), and polyethylene glycol (PEG).

2.3. LC-MS/MS analysis

All MS/MS analyses were performed under identical conditions using an UltiMate 3000 RSLCnano system (Thermo Scientific) on-line coupled with a HCTultra PTM Discovery System ion trap mass spectrometer equipped with a nanospray (Bruker Daltonik). After injection (10 out of the total of 12 \(\mu\)L present in the sample vial), peptides were concentrated and desalted on a trap column (75 \(\mu\)m ID × 150 mm length; 300 nL min \(^{-1}\)) filled with Acclaim PepMap RSLC C18 (Dionex).

3. Results and discussion

Our results indicate that significant peptide losses occur immediately after placing the peptide sample into the autosampler vial or, possibly, within the LC system during sample injection and loading on the trap column (Fig. 1). We propose the use of PEG (0.001%) which effectively decreases sample losses probably due to competition in adsorption with the peptides and it has no adverse effects to chromatographic separation or LC-MS system (2 years of continuous use).

Type of the vial material did not play as crucial role as compared to SIS composition especially in combination with PEG (see Fig. 2). Commonly available PP vials were selected for further experiments as one of the best of the tested materials.
In contrast to the model sample, time of storage played more important role in case of FA solution. While the number of identified peptides did not change dramatically over the tested time period for samples in U/T and PEG, the number of peptides was reduced by 34% and 44% after 24 h and 48 h of storage using FA, respectively. To assess the nature of peptides being likely to be preserved in the solution in presence of particular SISs, we investigated their retention times. In general, peptides are eluted by reversed-phase chromatography according to their increasing hydrophobicity. We observed significant decrease in the number of hydrophobic peptides (peptides with RT > 25 min) in FA after 24 h in comparison with the other two solutions (see Fig. 3).

4. Conclusions

Composition of sample injection solution was found to play important role even if samples were immediately analyzed. Compared to other tested additives, PEG represents a simple and cheap solution improving the overall coverage of real sample. No substantial
quantitative losses up to 48-hours storage under studied conditions and no compromising of the LC-MS/MS system performance was observed. We therefore propose the use of 0.001% PEG as an additive into sample injection solution for efficient reduction of adsorptive losses of peptides in autosampler vials.

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REFERENCES

PRACTICAL ASPECTS OF COMPLEXATION OF BUFFER CONSTITUENTS WITH NEUTRAL COMPLEXATION AGENTS IN CAPILLARY ELECTROPHORESIS

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Summary

The complexation of buffer constituents with the complexation agent present in the solution can very significantly influence the buffer properties, such as pH, ionic strength or conductivity. These parameters are often crucial for selection of the optimal separation conditions in analytical separation techniques, particularly for capillary electrophoresis. We demonstrate that even commonly used buffers significantly complex with usual chiral selectors as neutral cyclodextrins. This type of complexation and the subsequent change in properties of the buffer can have substantial practical aspects in capillary electrophoresis; namely on determination of complexation constants, development of system peaks and last but not least can deteriorate the results of electrophoretic separation.

1. Introduction

Capillary electrophoresis (CE) is a widely employed separation technique. It offers many useful modifications that make use of the presence of complexation agents in background electrolyte (BGE). The fact that interactions between analytes and complexation agents are reflected in changes of electrophoretic behavior of the respective compounds can be advantageously used to determine complexation constants or for other studies of non-covalent binding in chemistry or biology. The complexation of analyte(s) with complexation agent(s) is described in detail in the literature nowadays. However, possible changes of the BGE properties due to the complexation of buffer constituents with a neutral complexation agent are mentioned rarely. Just a few authors proposed to control the pH of the buffer after the addition of a ligand.

2. Results and discussion

The complexation of buffer constituents can result in substantial changes in buffer properties. The impact of complexation of buffer constituents with a neutral complexation agent can be demonstrated theoretically using our software Simul 5 Complex as well as experimentally for common buffers. We were able to observe the significant pH changes for the buffers frequently used in separation techniques, see Fig. 1.

The substantial changes of pH also have the practical impact on electrophoretic results; namely complexation constant determination, system peak development and proper separation of analytes. As the result, the complexation parameters determined in the interacting buffers cannot be regarded as thermodynamic ones and may provide misleading information about the strength of complexation of the compound of interest.

Fig. 1. Shifts in pH (ΔpH) after addition of 10 ± 0.5 mM α-CD (cyan), β-CD (green), HP-β-CD (blue) or DM-β-CD (magenta) in six commonly used buffers and the model system (benzoate buffer). Groups of columns are marked by the name of the buffering compound and pH value of the original buffer (without addition of CD). Error bars represent standard deviation of the measured value.
We also demonstrate that the development of system peaks in interacting buffer systems significantly differs from the behavior known for non-complexing systems, as the mobility of system peaks depends on the concentration and type of neutral complexation agent. Finally, we show that the use of interacting buffers can totally ruin the results of electrophoretic separation because the buffer properties change as the consequence of the buffer constituents’ complexation, see Fig. 2.

4. Conclusions

We demonstrate that complexation of buffer constituents with neutral complexation agents has to be always considered in selection of optimal separation conditions or determination of complexation constants in capillary electrophoresis. Complexation of buffer constituents changes the fundamental properties of the buffer such as pH, ionic strength or conductivity. Thus, we recommend check pH of the buffer solution before and after addition of neutral complexation agent to exclude the effect of complexation of buffer constituents on the electrophoretic results.

The support of the Grant Agency of the Czech Republic, Grant No. P206/12/P630, Grant Agency of Charles University, Grant No. 323611, the project Kontakt LH11018, and the long-term research plan of the Ministry of Education of the Czech Republic (MSM0021620857), are gratefully acknowledged.

REFERENCES

MALDI MS AND SALD ICP MS: COMPLEMENTARY DETECTION TECHNIQUES FOR METALLOPROTEOMICS

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Summary

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and substrate-assisted laser desorption inductively coupled plasma (SALD ICP) MS are proposed as complementary detection techniques that allows acquisition of both molecular and element specific information from a single capillary electrophoresis (CE) run recorded on a custom-designed sample target. The whole concept is demonstrated on the analysis of rabbit-liver metallothionein (MT) isoform mixture.

1. Introduction

Metals play a crucial role in physiology and pathology of biological systems. It has been estimated that the metalloproteins encompass about one third of all proteins. Therefore, the studies focused on the comprehensive characterization of the metal-protein or metal-metabolite complexes are becoming increasingly important.

In this contribution, we present a novel approach for comprehensive multidimensional analysis of metalloproteins. This method is based on an off-line coupling of a single CE run to both MALDI MS and SALD ICP MS. The effluent fractions are collected on an Au-coated polyethylene terephthalate glycol (PETG) sample plate that is compatible to both MS methods.

2. Experimental

CE was carried out in uncoated fused silica capillaries (Polymicro Technologies, USA) of 70 cm (length) and 75 μm (i.d.) using a laboratory-built system. MT isoforms were separated in 20-mM NH₄HCO₃/HCOOH, pH 7.4 at 20 kV. The samples were injected hydrodynamically from unbuffered solution. A fiber optic UV detector was used for the immediate monitoring of the separation process. Fraction collection for the following MALDI MS/SALD ICP MS analyses was carried out using a liquid junction interface and sub-atmospheric deposition chamber. CE fractions were collected on a PETG sample plate coated with 5-nm Au layer, and covered with MALDI matrix, 26.5 mM α-cyanohydroxycinnamic acid (CHC), in 50% ACN and 1% TFA or 65 mM 2,5-dihydroxybenzoic acid (DHB) in 50% ACN and 0.05% ammonia solution. MALDI mass spectra were acquired using MALDI TOF/TOF mass spectrometer (Autoflex Speed, Bruker Daltonics). Afterwards, the Au-PETG sample plate was inserted into an ablation system (model UP 213, New Wave Research, USA) and SALD ICP MS was performed with ICP mass spectrometer (model 7500 CE, Agilent Technologies, USA).

3. Results and discussion

A novel approach (see Fig. 1 for method workflow) for complementary molecular and element-specific detection that combines two methods developed previously, CE MALDI MS (ref.3) and CE-SALD ICP MS (ref.4), is proposed. The compatibility of the both MS methods is ensured by the use of a PETG sample plate coated with a golden nanolayer for fraction collection. While the PETG acts as an efficient substrate for SALD ICP MS, the Au nanolayer provides necessary conductivity for MALDI time-of-flight MS. The optimal Au-layer thickness was determined to be 5 nm. Overall performance of the sample plate for the both MS methods as well as the effect of MALDI MS on consequent SALD ICP MS measurement was investigated and the separation and deposition conditions were optimized.

MT mixture (14 ng, corresponding to 2 pmol in total) was injected and separated under the optimal conditions. Fractions at migration time \( t_{mig} = 7–12 \) min were collected in 2-s intervals on the Au-PETG target, and covered with

Fig. 1. Experimental workflow of the CE(UV)-MALDI MS/SALD ICP MS
the MALDI matrix solution. The separation record was then subjected to consecutive MALDI MS and SALD ICP MS analyses. The UV-trace, MALDI MS and ICP MS electropherograms recorded from a single separation are shown in Fig. 2.

To take full advantage of the off-line coupling potential, two different MALDI matrices, CHC and DHB in acidic and neutral pH, respectively, were applied on the adjacent spots corresponding to the MT peaks. Thus additional information can be obtained: While the information about protein apoforms is revealed under acidic condition, the un-dissociated metal-protein complexes can be detected at neutral pH.

4. Conclusions

A novel method for metalloprotein analysis based on off-line coupling of capillary electrophoresis to both MALDI MS and SALD ICP MS is proposed. We believe it represents a viable alternative to common on-line coupling which employs electrospray ionization and nebulizer ICP MS. The off-line hyphenation allows decoupling separation and both detection processes in time and space and offers further options, such as re-analysis or archiving of the separation record, laser-induced fluorescence detection or on target protein digestion.

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REFERENCES

Summary

Capillary electrophoresis coupled to mass spectrometry (CE-MS) posses powerful tool for separation and detection of wide range of ionic species. To avoid complicated constructions of interfaces we have conducted the separation within a long and thin nanoelectrospray tip. We have investigated main properties of such an experimental design on a complex sample of tryptic digest of cytochrome c.

1. Introduction

This work is aimed at optimization of electrophoretic separation within a long nanoelectrospray tip with mass spectrometric detection. The potential drop needed for sufficient resolution was reached by using system with high resistance.

2. Experimental

The electrophoretic separation was performed in 100 cm × 10 μm I.D. silica fused capillary ended by sharp polished ESI tip. The ESI tip was positioned approximately 2 mm in front of the mass spectrometer sampling orifice (LTQ Velos Pro, Thermo Fisher).

The sample of tryptic digest of cytochrome c from equine heart (Sigma Aldrich, ≥ 95%) was prepared by common procedure. To make solution free of ammonium bicarbonate used as a buffer it was evaporated at 37 °C and dissolved again in 0.01% formic acid. Sample of concentration 0.12 mg mL⁻¹ was loaded from a gas pressurized chamber used also to assist the liquid flow inside the capillary during the experiments with nanoelectrospray. As a background electrolyte 0.01% HCOOH was used.

3. Results and discussion

At our simple experimental design of interfacing a voltage is applied only at the beginning of the capillary and there is no direct control over the voltage at capillary end, which is ended by a nanoelectrospray tip.

If worked with this type of interface, it is the most challenging to adjust sufficient potential drop within a separation channel and reach optimal voltage on the nanoelectrospray tip at the same time. To meet such conditions it is crucial to work with system of high resistance.

Conductivity of background electrolyte is one of the most important factor influencing resistance. We investigated wide range of potential background electrolytes and eventually 0.01% HCOOH (σ = 265 μS cm⁻¹, pH 3.20) has been chosen as the most appropriate. Not only that it has low conductivity but it still has acidic pH which is desirable for charging of analytes.

For further increase of resistance capillary of I.D. 10 μm was used. At the end of the capillary sharp tip was fabricated. There is several ways for fabrication of nanoelectrospray tip (e.g. pulling, etching and grinding). The most promising results were obtained for grinded tips. However, thin capillary brings more than high resistance. Other upsides are ability to create fine electrospray plume, low theoretical plate is reached and it posses more robust system for electrospraying.

Velocity of ions during the separation is given by contribution of hydrodynamic flow rate and electrophoretic mobility. To reduce the first named contribution the separation was run for 12 minutes at voltage of 30 kV with no flow rate. Later the conditions were changed to stable plume could be formed. Voltage was decreased to 5 kV and flow rate of 5 nL min⁻¹ was developed (Fig. 2).

At such experimental conditions we have reached promising resolution and sensitivity. What is more loaded amount of sample was only 0.93 fmol, which predestine this type of interfacing for analysis of small amount of sample.

Fig. 1. Scheme of used interfacing
4. Conclusions

On a model sample of tryptic digest of cytochrome c was shown that this type of interfacing is viable. In comparison to other approaches it brings construction simplicity and potential to work with small amount of sample.

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REFERENCES

APPLICATION OF PRESSURIZED HOT WATER FOR ETCHING OF MICROFLUIDIC STRUCTURES

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Summary

The subject of this study is a new method of glass etching and its application in microfluidics. Commonly, hydrofluoric acid is used for glass etching. In this study we have tested a new method of glass etching using pressurized hot water. Test motives were prepared using photolithography and metal sputtering on the glass substrate and the etching was performed in a newly developed device capable to compress and heat water to its critical point. Electron microscopy and profilometry have been used for analyzing the surface of the etched glass substrate.

1. Introduction

Commonly, hydrofluoric acid is used for glass etching. However, its toxicity represents a significant health and environmental risk factor. This study focuses on the development of a new etching procedure using the recently developed device with temperature controlled high pressure chamber, providing a wide range of pressures and temperatures up to the critical point of water. Glass chips with metallized surface were prepared and the test pattern was photolithographically etched in the metal protection layer prior to the glass etching. Series of experimental data obtained under different pressures, times and temperatures were evaluated by electron microscopy and optical profilometry.

2. Experimental

2.1. Testing of new etching method

The glass slides were sputter coated by a 200 nm layer of Cr followed by spincoating a 1 µm layer of a positive photoresist. The test pattern was created by a direct laser writer (Heidelberg Instruments µPG 101) on 48 glass substrates. After etching by hot water the surface structures of all substrates were analyzed by electron microscope Helios Nanolab 600i (FEI, Brno) and in an optical profilometer ContourGT – X8 (Bruker, FRG).

2.2. Application of new etching method

The object of the second part was to produce a fully enclosed microfluidic chip using the previously described etching protocol. Suitable conditions for etching were chosen, according to the results from surface structure analysis, and the cover glass was bonded to the etched substrate in the oven (Řevnice, Classic – 3013-S) at 600 °C. Finally, the chip was cut using Precision CNC Dicing / Cutting Saw SYJ-400 supplied by MTI Corporation (USA).

3. Results and discussion

The structure analysis showed that the surface properties of the etched substrate were changing depending on either etching pressure or etching temperature.

3.1. Dependency on etching pressure

The lines accompanied with arrows define the width (w) of underetching. With growing pressure, the substrate is more underetched. The etching water pressure had no
influence on the channel depth; however, the surface roughness was highly influenced by the etching temperature as shown in Fig. 2. The difference of 40 °C made the surface of substrate 6 more porous. Furthermore, the etching water temperature influenced the channel depth, with growing temperature, the channels were deeper.

3.2. The final microfluidic device

After evaluation of the etching parameters a fully enclosed microdevice was prepared by thermal bonding of the cover glass on top of the substrate with the etched microchannel. Fig. 3 shows both the channel structure and the final microfluidic chip after dicing.

A suitable (porous) channel surface (second picture of Fig. 3) was chosen for the etching of the microfluidic device that can be used in further research e.g. for immobilization of enzymes such as trypsin.

4. Conclusions

The achieved results prove that the new glass etching method allows substituting the HF based toxic process by the eco-friendly hot water etching. The developed protocol enables good control of the surface properties and the new etching method may become a viable alternative to commonly used methods.

This project is co-financed by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182). The support of the Grant Agency of the Czech Republic (P206/12/G014) and the institutional research plan (RVO 68081715) are also gratefully acknowledged.

REFERENCE

AN INSIGHT INTO THE RETENTION MECHANISM ON CYCLOFRUCTAN-BASED CHIRAL STATIONARY PHASES

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Summary

Cyclofructan-based chiral stationary phases were shown as a promising possibility for separation of chiral compounds in HPLC. In this work retention mechanisms of cyclofructan-based chiral stationary phases are described. Additionally, the retention mechanism of dimethylphenyl-carbamate cyclofructan 7 was studied under the conditions of supercritical fluid chromatography. A linear free energy relationship model was utilized for characterization of retention interactions.

1. Introduction

In 2009 Armstrong et al. introduced chiral stationary phases (CSPs) based on native and derivatized cyclofructans (CFs) (ref.1). Since that time CFs as chiral selectors found applications in many separation techniques including HPLC, GC, and CE (ref.2–5). The structure of CF molecule is composed of six, seven or eight D-fructofuranose units connected via \( \beta(2 \rightarrow 1) \) linkage 6. The connected saccharide units form a crown-ether ring, which significantly determinates chromatographic behavior of CFs. Common abbreviations CF6, CF7 and CF8 indicates a number of saccharide units forming the ring. Native CFs have their crown-ether skeleton sterically blocked by intramolecular hydrogen bonds, therefore their separation abilities are quite limited. As a result of introducing of derivatization groups into the CF molecule the CF molecule relaxes and crown-ether core becomes more accessible and the enantioselective potential significantly increases. Character of the derivatization moiety has substantial impact, since aliphatic derivatization moieties particularly improve enantioselectivity especially for amines, while aromatic derivatization groups improve enantioselectivity among various compounds.

The aim of our work was to reveal retention interaction occurring on CF-based CSPs, namely isopropyl carbamate CF6 (IP-CF6 CSP), R-naphthylethyl carbamate CF6 (RN-CF6 CSP) and dimethylphenyl carbamate CF7 (DMP-CF7 CSP) under the conditions of normal phase mode HPLC and last mentioned CSP also under the condition of supercritical fluid chromatography (SFC). In the first article introducing CF-based CSPs was mentioned the possibility of use CF-based CSPs in SFC. However, the detailed study has never been done.

2. Experimental

To determine and quantify the interactions participating in the retention process a linear free energy relationship model (LFER) was employed. This model is based on the idea that retention (expressed as \( \log k \), where \( k \) is a retention factor) is composed of independent contribution according to the involved interaction type. The most common LFER equation works with five type of interactions (dispersion interactions, hydrogen bond acidity, hydrogen bond basicity, polarity/polarizibility, and ability to interact via \( n- \) and/or \( \pi- \) electron pairs). The LFER set contained 35 compounds, whose chemical features were described. The description is based on the physical measurements/calculations and expressed as a number (a descriptor). After analysis of LFER set, logarithms of obtained retention factors undergo multilinear regression analysis (\( \log k \) against the descriptors). Results of multilinear analysis, regression coefficients, indicate whether the particular interaction type is dominant in the stationary phase (positive value of the regression coefficient) and thus increases the retention or in the mobile phase (negative value of the descriptor) and decreases retention or the same in both phases (statistically insignificant regression coefficient). Despite the fact that LFER model does not take into account the phenomena of chirality, it provides valuable information about the interaction mechanisms.

3. Results and discussion

At first, individual CF-CSPs were compared (see Table I). The same types of interactions in a different extent were shown to be preferred by all three CSPs, i.e. hydrogen bond acidity and dipolarity/polarizibility. Also the effect of hydrophobicity as the retention reducing factor plays a role with all tested CF-based CSPs. Hydrogen bond basicity and interactions with \( n- \) and \( \pi- \) electron pairs seemed to be insignificant.

The same model as for HPLC was used for the characterization and further comparison of retention interaction on DMP-CF7 CSP under HPLC and SFC conditions. LFER model confirmed that different interactions participated to different degrees in the retention process on DMP-CF7 CSP in SFC and HPLC.
Table I  
Comparison of regression coefficients of the LFER equation and correlation coefficient $R$ for systems with three CF-based CSPs under normal phase mode HPLC

<table>
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<th>Column</th>
<th>Mobile phase</th>
<th>Model</th>
<th>$e$</th>
<th>$s$</th>
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<th>$b$</th>
<th>$v$</th>
<th>$c$</th>
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<td>hex/IPA/TFA</td>
<td>O.M.</td>
<td>x</td>
<td>1.128</td>
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<td>0.3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

CI represents ±95% confidence interval. x, insignificant interaction; O.M., optimal model of the LFER equation; $p$, statistical $p$-value. The $p$-values express probability of the error that the individual coefficient does not contribute to the model, i.e., $p$-values represent the significance of the individual coefficients.

Table II  
Comparison of regression coefficients of the LFER equation and correlation coefficient $R$ for DMP-CF7 CSP in SFC and HPLC

<table>
<thead>
<tr>
<th>Method</th>
<th>Mobile phase</th>
<th>Model</th>
<th>$e$</th>
<th>$s$</th>
<th>$a$</th>
<th>$b$</th>
<th>$v$</th>
<th>$c$</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>Hex/IPA/TFA</td>
<td>O.M.</td>
<td>x</td>
<td>0.789</td>
<td>x</td>
<td>1.597</td>
<td>-0.810</td>
<td>-0.881</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80:20:0</td>
<td>± 95% CI</td>
<td>0.277</td>
<td>0.310</td>
<td>0.389</td>
<td>0.330</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>Hex/IPA/TFA</td>
<td>O.M.</td>
<td>x</td>
<td>0.933</td>
<td>x</td>
<td>1.516</td>
<td>-0.917</td>
<td>-0.940</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80:20:0/0.5</td>
<td>± 95% CI</td>
<td>0.254</td>
<td>0.274</td>
<td>0.348</td>
<td>0.296</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>SFC</td>
<td>CO2/IPA/TFA</td>
<td>O.M.</td>
<td>0.459</td>
<td>0.514</td>
<td>x</td>
<td>0.949</td>
<td>-1.011</td>
<td>-0.410</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80:20:0/0.0</td>
<td>± 95% CI</td>
<td>0.250</td>
<td>0.263</td>
<td>0.251</td>
<td>0.494</td>
<td>0.259</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>SFC</td>
<td>CO2/IPA/TFA</td>
<td>O.M.</td>
<td>0.537</td>
<td>0.472</td>
<td>x</td>
<td>0.936</td>
<td>-1.204</td>
<td>-0.227</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80:20:0/0.5</td>
<td>± 95% CI</td>
<td>0.253</td>
<td>0.267</td>
<td>0.255</td>
<td>0.501</td>
<td>0.364</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
<td>0.212</td>
<td></td>
</tr>
<tr>
<td>SFC</td>
<td>CO2/MeOH/TFA</td>
<td>O.M.</td>
<td>0.557</td>
<td>0.495</td>
<td>0.891</td>
<td>1.020</td>
<td>-0.806</td>
<td>-0.685</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95:5/0.0</td>
<td>± 95% CI</td>
<td>0.308</td>
<td>0.351</td>
<td>0.279</td>
<td>0.734</td>
<td>0.512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.001</td>
<td>0.007</td>
<td>0.000</td>
<td>0.033</td>
<td>0.010</td>
</tr>
<tr>
<td>SFC</td>
<td>CO2/MeOH/TFA</td>
<td>O.M.</td>
<td>0.627</td>
<td>0.553</td>
<td>0.795</td>
<td>1.113</td>
<td>-1.165</td>
<td>-0.422</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95:5/0.1</td>
<td>± 95% CI</td>
<td>0.446</td>
<td>0.508</td>
<td>0.404</td>
<td>0.455</td>
<td>1.064</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>v/v/v</td>
<td>$p$-value</td>
<td>0.007</td>
<td>0.034</td>
<td>0.000</td>
<td>0.033</td>
<td>0.254</td>
</tr>
</tbody>
</table>

CI represents ±95% confidence interval. x, insignificant interaction; O.M., optimal model of the LFER equation; $p$, statistical $p$-value. The $p$-values express probability of the error that the individual coefficient does not contribute to the model, i.e., $p$-values represent the significance of the individual coefficients.
(see Table II). The results suggested that the adsorption of some components of the mobile phases is more important in SFC than in HPLC. The lower content of alcoholic modifier in the mobile phase, the higher the adsorption, which significantly changes the characteristics of the separation system in SFC.

4. Conclusions

The LFER model was used to describe interactions participating in the retention and separation process on the CF-based CSPs in normal mode HPLC and on DMP CF7 CSP in SFC. Although LFER does not take into account chirality and spatial arrangement of analytes, it proved which forces take part in the interaction mechanism. The same types of interactions in a different extent were shown to be preferred by all three stationary phases, i.e. hydrogen bond acidity and dipolarity/polarizibility in HPLC. Also the effect of hydrophobicity as the retention reducing factor plays a role with all tested CF-based CSPs. Hydrogen bond basicity and interactions with \( n \)- and \( \pi \)-electron pairs seemed to be insignificant. Furthermore, the LFER model confirmed the different distribution of interactions influencing the retention process on DMP-CF7 CSP in SFC and HPLC.

The Grant Agency of the Charles University in Prague, project No. 356411, the Ministry of Education, Youth and Sports of the Czech Republic, project KONTAKT AM 2010 project LH11018, and the long-term project MSM0021620857 are gratefully acknowledged for the financial support. Furthermore, Prof. Ch. Roussel and Dr. N. Vanthuyne from the Chirosciences team of Aix-Marseille University are thanked for their kind help during the SFC experiments.

REFERENCES

1. Introduction

The homogenization of surface properties in multicomponent or hybrid fluidic devices, integrating silicon, glass, metals, photoresists (e.g., SU8) and elastomers (e.g., polydimethoxysilane, PDMS) requires the innovations of universal techniques which are independent of the nature of surfaces. It was recently reported that dopamine (DOPA) spontaneously oxidizes under alkaline conditions, and forms highly reactive products, that further forms thin-layer coating which can be additionally modified with various molecules of interest. This inspired a new strategy in surface science, namely mussel-inspired surface modification, regardless of the structural similarity between DOPA and catecholic compounds existing in mussel proteins. In this study, amine terminated polyacrylamide (AmPAM) was end-tethered on different fluidic materials (Si, glass, metals, SU8, PDMS) via mussel inspired surface modification in a one-pot approach, that is easily applicable to nano- and microfluidic devices. Modified surfaces were thoroughly characterized. It was found that the bio-antifouling DOPA-AmPAM surfaces were formed on any substrates with very high homogeneity. These findings provided important applications in nano- and micro-fluidic systems.

2. Experimental

The widely used materials for microfluidics such as SiO₂, glass, Cu, Au, SU8, and PDMS were modified with AmPAM by one-pot mussel inspired surface modification. The surfaces of interest are simply modified when in contact with an alkaline buffer (50 mM Tris.HCl, pH 9.0) solution that contains DOPA (2 mg mL⁻¹), AmPAM (1 mg mL⁻¹) and ammonium persulfate (1 mg mL⁻¹). AmPAM (MW of 15 100 Da) was synthesized and well characterized in our laboratory. We also modified various surfaces with DOPA coatings only. In general, a freshly prepared solution of DOPA with or without the acrylamide chain is injected into a microfluidic channel and/or flat substrates are immersed into this solution for several hours, then the channels are flushed (and the flat substrates rinsed) with deionized water and dried in a nitrogen stream prior the use or further characterization.

3. Results and discussions

3.1. Water contact angle (WCA)

The static WCA measurements (Fig. 1) show that the DOPA coating adhere to all employed wetting surface, and that independently of the value of the WCA before modification, all the materials become alike (θ = 50.5–53.9°) after the surface coating. This value decreases (θ = 31.5–36.6°) when AmPAM is added to the DOPA solution prior contact with the solid material, suggesting that the polyacrylamide chains get end-tethered onto the surface.

![Fig. 1. Static water contact angle on various surfaces. In blue are shown water contact angles for unmodified surfaces, in red for DOPA only modified surfaces and, in green, for DOPA-AmPAM modified surfaces](image-url)
most probably via the primary amino group. In case of very hydrophobic PDMS surface (θ₀ >110°), a significant decrease in WCA was also observed, but at much higher values, comparing to the other surfaces (θ_DOPA=92.8° and θ_AmPAM-DOPA=80.6°).

3.2. Oxidation kinetics and roughness

The most important factor that controls the surface morphology and uniformity is the dopamine oxidation kinetics[4-8] that can be influenced by the initial dopamine concentration, the pH of employed buffer and the presence of an oxidizing agent. It has been reported recently[7] that highly homogeneous, thin layer films are formed at the higher oxygen concentration via accelerated reaction kinetics. This inspired us to use an oxidizing agent to promote oxidation of dopamine in order to achieve thin films with high uniformity.

Dopamine consumption was determined from UV spectra for the three different conditions (air, oxygen and ammonium persulfate initiation). Surprisingly, the complete dopamine consumption (~ 98 %) was observed after 1h of oxidation in the presence of ammonium persulfate, whereas the highest dopamine consumption was only 38 % in the air bubbled and 60 % in the oxygen bubbled solution. These results suggested that the oxidant induced oxidation of dopamine is irreversible reaction instead of reversible reaction as in case of pH induced oxidation (air and oxygen bubbling).

AFM measurements were performed to study morphology of the surface of AmPAMDOPA as well as DOPA deposited films on silicon oxide surface. Modified substrates in air and bubbled oxygen were also characterized. AFM images (Fig. 2) showed that all modified surfaces are well-covered. The same results were obtained by cyclic voltammetry curves of DOPA and DOPA-AmPAM modified gold electrodes, no redox peaks of electroactive probe was observed in accordance with the shielding effect by well-covered organic films. The thickness of AmPAM-DOPA after 3 h of deposition determined from AFM to be around 10 nanometers.

The root mean square roughness was used to evaluate roughness of deposited coating. We observed the significant decrease in roughness of organic films in the presence of ammonium persulfate. The roughness of AmPAM-DOPA and DOPA are determined to be 1.05 and 0.96 nm, instead of 2.44 and 1.92 nm in air, or 1.63 and 1.17 nm in bubbled oxygen. Alternatively, the density of aggregation considerably decreased in addition of oxidant (only several 102 nm sized grains on 10 μm²). It was

Fig. 2. AFM images of unmodified and modified silicon oxide surfaces
believed that the deposition of films containing DOPA is initiated by adsorption of oxidized radicals (mostly 5,6-dihydroindole and its derivatives) on surfaces\(^4\), then continues by curing process in which the cross-linking net of these molecules are formed through either covalent binding\(^8\) or non-covalent binding\(^9\). The fact is the use of oxidant created more initial radicals and the surfaces became more uniform with a decreased size of aggregates. Consequently, these organic depositions containing DOPA are spontaneously formed in a densely structural state on surfaces. This explains the important increase in homogeneity of modified surfaces in the presence of oxidant.

3.3. Protein adsorption

The non-fouling properties of our surfaces were studied with fluorescein (FITC)-albumin conjugate. The unmodified and modified PDMS surfaces were dipped into the FITC-albumin solution (1 mg mL\(^{-1}\) in PBS pH 7.4) and incubated at room temperature for 18 hrs. After the incubation was completed, the surfaces were washed with deionized water and dried in a stream of nitrogen. Compared to bare and DOPA modified PDMS surfaces, the fluorescence was dramatically decreased on the DOPA-AmPAM surface (Fig. 3), suggesting that the hydrophilic PAM brush is necessary to minimize the protein adsorption.

4. Conclusions

We have demonstrated the versatility of our approach for the modification and functionalization of surfaces of any solid material. In this work, we were interested to exploit the non-fouling properties of the modified surfaces prepared from hydrophilic polymer brushes. We applied our strategy to the modification and homogenization of surfaces inside nano- and microfluidic channels. The advantage of the developed strategy is inherited in the substrate independent chemistry and low viscosity of the modifying medium. The formation of DOPA and DOPA-AmPAM films on all the studied materials was clearly demonstrated in our work. The adsorption studies with the FITC-albumin conjugate showed that the adsorption was dramatically decreased on the PDMS-DOPA-AmPAM substrate due to the presence of the PAM polymer brushes.

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REFERENCES

INFORMATION ENTROPY APPROACH AS A METHOD OF ANALYSING BELOUSOV-ZHABOTINSKY REACTION WAVE FORMATION

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Summary

This work aims to develop a method of analysis for self-organized systems such as living cells culture or herds in native ecosystem. In the selection of the appropriate model was chosen a simpler approach – the Belousov-Zhabotinsky reaction (chemical clock).

Proposed method based on the information theory of multifractal objects. We use the Renyi information entropy equation for calculation of information gain by which a point contributes to the total information in the image, the point information gain. Obtained values present unique information about object structure. Method allows highlight tiny features in investigated sample structure and characterizes system behavior in dynamic.

1. Introduction

The Belousov-Zhabotinsky (BZ) reaction was devised as a primitive model of citric acid cycle. When performed in a thin – few centimeters thick – layer, it creates easily observable travelling waves which may be captured by ordinary colour camera and analysed. The system behavior in time indicates existence of a sequence of distinct states stable for certain period of time. The experimenter has control of mechanical constraints imposed on the system. States in the course of the reaction may be identified in all cases when the geometry of the experimental vessel allows creation of travelling waves.

2. Material and methods

Experiments were performed with the oscillating bromated-ferroin-bromomalonic acid reaction type (kit were provided by Dr. Jack Cohen). The reaction mixture was composed out of following solutions: 0.34 M sodium bromate, 0.2 M sulphuric acid, 0.057 M sodium bromide, 0.11 M malonic acid as substrate and redox indicator 0.12 M 1,10-phenantroline ferrous complex. All reagents were coherently mixed under temperature 22 °C and added into Petri dish. Images were captured by Nikon D90 camera in regime Time lapse shooting in interval 10 second between snapshots.

In our practical approach\(^2,3\) we calculate the Renyi entropy contribution of each of the points in the image. We calculate the Renyi entropy difference for the data set containing the examined point and the dataset in which the examined point was excluded. This is the Point Information Gain \((\gamma_\alpha(x,y))\) for given entropy of the order \(\alpha\):

\[
\gamma_\alpha(x,y) = \frac{1}{1-\alpha} \ln \left( \frac{\sum_{i=x}^{p_x} \rho_{i,x,y}^\alpha}{\sum_{i=x}^{p_x} \rho_i^\alpha} \right) - \frac{1}{1-\alpha} \ln \left( \frac{\sum_{i=y}^{p_y} \rho_{i,x,y}^\alpha}{\sum_{i=y}^{p_y} \rho_i^\alpha} \right)
\]

(1)

where \(\rho_{i,x,y}\) and \(\rho_i\) are probabilities of occurrence of given intensity for given point \(x, y\) coordinate of camera pixel at given \(\alpha\) in the image without and with the examined point.

In the next step number of points of given intensity is summed and normalized – we obtain Point Information Gain Entropy \((H_\alpha)\):

\[
H_\alpha = \sum_{x=1}^{p_x} \sum_{y=1}^{p_y} \gamma_\alpha(x,y)
\]

(2)

And on the final step of image processing were calculated Point Information Gain Entropy Density \((\Xi_\alpha)\):

\[
\Xi_\alpha = \sum_{i=1}^{p_x} \gamma_\alpha(x,y)
\]

(3)

For given dataset, i.e. image, the ordered set of \(H_\alpha\) and \(\Xi_\alpha\) tuples a unique characteristic of the image, i.e. each two different images will have different sets, provided that the image is captured with infinite precision and the calculation is performed for all \(\alpha\) values form 0 to infinity.

In real case the precision is defined by the digital camera and the set of \(\alpha\) values is chosen arbitrarily.

Using statistical approaches such as principal component analysis (PCA)\(^4\) we may construct orthogonal spaces which best fit the observed dataset. All data divided on the clusters depend on the value of the PCA-components. Each cluster of the trajectory present the one of the states of the BZ reaction.

3. Results and discussion

The set of \(H_\alpha\) were used for describing the evolution of the system as a multi-fractal object. The state evolution is split in logical sequence of clusters in the new orthogonal, although still phenomenological, state space. On the Fig. 1 is shown decomposition of the system trajectory of the Belousow-Zhabotinsky reaction into series of states which are for distinct period of time.
asymptotically stable under current conditions. Clusters (series of images) are very well separated and consistent in time, for each of group could find characteristic image represented the state of the system in their developing process. Moreover, each of the clusters states should have its own spectrum of $H_\alpha$ values which characterizes it.

4. Conclusions

Applying the information entropy as the basic characteristics of the image is a promising area for further research, with the ultimate aim of which is to create the reliable method of automated segmentation of the self-organizing system state space obtained by non-invasive imaging method. And the trajectory segmentation may be used despite to the fact that we do not know the proper manifold in the internal orthogonal coordinate space.

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REFERENCES

1. Dr. Jack Cohen at http://drjackcohen.com/BZ01.html.

Fig. 1. The state trajectory of the BZ reaction performed in the Petri dish. Principle component analysis of the $H_\alpha$ allows to designate different states of the system (changes in waves structure during the reaction evolution), from the point of view of the method clusters of points in the state space. Scores differ significantly between clusters oriented and form logical trajectory in the principal coordinates space.