Abstract

The presented dissertation thesis focuses on the detailed characterization and utilization of various modes of liquid chromatography with multiple interaction mechanisms for analysis of biologically active compounds, such as peptides, proteins or therapeutic oligonucleotides. In the first part of the thesis, the retention/interaction mechanisms of selected reversed-phase and mixed-mode stationary phases were investigated thoroughly, with a strong emphasis on describing electrostatic interactions. For their detailed characterization, new method using pure aqueous mobile phases with different pH values and model permanently charged analytes (including inorganic anions) was developed. It's important to note that electrostatic interactions can be arise not only from the presence of ligands with ion-exchange properties (mixed-mode stationary phases) but also from residual dissociated silanols.

To describe the retention behavior of peptides and proteins, *i.e.* zwitterionic compounds, stationary phases with mixed-mode character were selected. The retention and peak shape in these systems are fundamentally affected not only by pH of aqueous part of the mobile phase, but also by the buffer concentration. With the aim of developing a simple method for on-line protein digestion, chromatographic columns with immobilized trypsin were characterized in terms of the effects of chromatographic conditions (temperature, pH of the mobile phase, flow rate, content of organic modifier in the mobile phase) on the activity of immobilized trypsin. Under optimized conditions, several proteins were on-line digested, followed by the separation of digests using mixed-mode column. The results were compared with off-line digestion using trypsin spin columns and, in the case of cytochrome C also with analysis of its commercially available digested standard.

Therapeutic phosphorothioate oligonucleotides were characterized and analyzed in two different modes, namely ion-pairing reversed-phase liquid chromatography and hydrophilic interaction liquid chromatography. Both systems do not provide separation of diastereomers under optimized conditions, which is crucial for their reliable analysis. In the first mentioned mode, the effect of the separation temperature, type and concentration of both the ion-pairing agent and its counterion on the suppression of diastereomeric separation and the resolution of oligonucleotides of different lengths (n and n-x) was comprehensively inspected. The discovered high potential of hydrophilic interaction liquid chromatography and its compatibility with mass detection were utilized for separation and identification of impurities and metabolites of the therapeutic oligonucleotide nusinersen.