

## Abstract

Glycoproteomics is a challenging branch of proteomics because of the micro- and macro-heterogeneity of protein glycosylation. Hydrophilic interaction liquid chromatography (HILIC) is an advantageous alternative to reversed-phase chromatography for intact glycopeptide separation prior to their identification by mass spectrometry. Nowadays, several HILIC columns differing in used chemistries are commercially available. However, there is a lack of comparative studies assessing their performance, and thus providing guidance for the selection of an adequate stationary phase for different glycoproteomics applications. Here, we compare three HILIC columns recently developed by Advanced Chromatography Technologies (ACE) with unfunctionalized (HILIC-A), polyhydroxy functionalized (HILIC-N), and aminopropyl functionalized (HILIC-B) silica with a C18 reversed phase column in the separation of human immunoglobulin G glycopeptides. HILIC-A and HILIC-B exhibit mixed-mode separation combining hydrophilic and ion-exchange interactions for analyte retention. Expectably, reversed-phase mode successfully separated clusters of immunoglobulin G1 and immunoglobulin G2 glycopeptides, which differ in amino acid sequence, but was not able to adequately separate different glycoforms of the same peptide. All ACE HILIC columns showed higher separation power for different glycoforms, and we show that each column separates a different group of glycopeptides more effectively than the others. Moreover, HILIC-A and HILIC-N columns separated the isobaric A2G1F1 glycopeptides of immunoglobulin G, and thus showed the potential for the elucidation of the structure of isomeric glycoforms. Furthermore, the possible retention mechanism for the HILIC columns is discussed on the basis of the determined chromatographic parameters.