Current Topics in Analytical Chemistry
Vol. 9, 2012

Original Communication

Evaluation of ZIC-HILIC columns for the analysis of flavonols

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ABSTRACT

Zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) was used to study the retention of selected flavonols. In gel ZIC columns the active layer grafted on polymeric support contains both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups in the molar ratio of 1:1, separated by a short alkyl spacer. Retention behavior of the compounds was investigated by varying the separation conditions, e.g. the kind of organic solvent in the mobile phase (acetonitrile or methanol), its ratio to water and pH value. Polar (hydrogen-bonding and dipole-dipole) interactions in the stationary phase are of primary importance, even though weak electrostatic interactions affect the separation of analytes carrying either positive or negative charges.

KEYWORDS: HILIC chromatography, ZIC columns, flavonols

1. INTRODUCTION

Hydrophobic interaction liquid chromatography (HILIC) is an interesting alternative for the analysis of polar substances, offering a complementary selectivity compared to reversed-phase liquid chromatography (RPLC). HILIC can be defined as a separation mode that combines stationary phases usually used in the normal phase (NP) mode and mobile phases used in the RP separations [1, 2]. HILIC, a term coined by Alpert [3], is based on the use of polar stationary phases combined with mobile phases containing a high organic content and a small amount of water. In aqueous-organic mobile phase, water is preferentially adsorbed on the surface of silica as well as other polar adsorbents forming a water-rich pseudostationary phase. Partitioning of polar compounds between a water-enriched layer partially immobilized on the surface of the stationary phase and a highly organic mobile phase is postulated to be primary retention mechanism [4-8]. Hydrogen bonding participates in this process, possibly as a driving force in the partitioning, while electrostatic interactions contribute to the retention mechanism to a varying degree, depending upon the nature of the stationary phase, the ionization of the analytes and the type and concentration of buffer salts in the mobile phase. In HILIC mode, the elution order is often the opposite of that obtained with a RPLC. In addition, HILIC has the advantage of enhanced detection sensitivity when used in conjunction with mass spectrometry due to high organic content of the mobile phase, which allows efficient spraying and desolvation in electrospray ionization [9, 10].

Silica is the most often employed chromatographic support for HILIC. A wide range of functional groups are chemically bonded to silica surface including aminopropyl, amide, cyano, diol and zwitterionic (ZIC) [11, 12]. In ZIC-HILIC columns the active layer grafted on wide pore silica or polymeric support contains both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups in the molar ratio of 1:1, separated by a short alkyl spacer. The positive charge is closest to silica surface, while the

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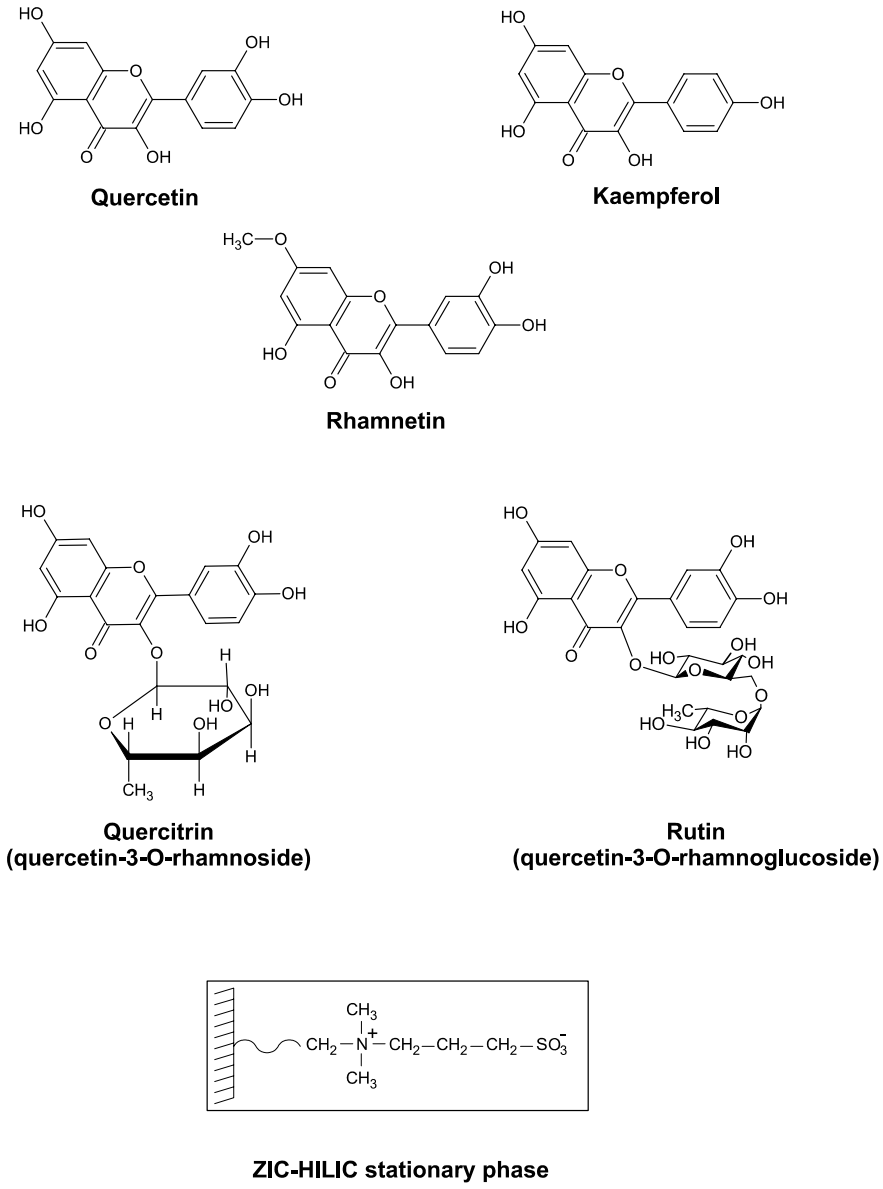

Fig. 1. Chemical structures of the studied compounds and ZIC-HILIC column.

negative charge is in the terminal end of bonded ligand and is thus more accessible for interaction with the analytes. A low net negative surface charge of the bonded layer is attributed to large distance of the sulfonic groups from the gel surface and it is affected only very little by pH.

In a typical HILIC mobile phase, acetonitrile is used as a weak eluent and water or aqueous buffer as a strong eluent. A minimum of 3% of water is essential for formation of the aqueous layer involved in the separation. The advantage of using acetonitrile over other organic solvents is its chemical structure, which does not favour the formation of hydrogen bonds and can avoid competition between solvent and water molecules for the stationary-phase interaction sites [13]. Liu *et al*. [14] proposed the use of ethanol as weak eluent alternatives to acetonitrile for the separation of hydrazines in order to allow the use of nitrogen chemiluminescence detector, which does not permit the presence of nitrogen in the mobile phase. Methanol gave separations with low retention

times and isopropanol presented both longer separation times and lower efficiencies.

The interest in the analysis of flavonols is continuously increasing due to the protective antioxidant role of these compounds in human body against cancer and coronary heart diseases. Flavonols, low polar compounds, are strongly retained in reversed phase chromatography using C-18 column. The typical time of RP analysis is in the range of 30-60 min with gradient elution. The objective of this work was investigating the effect of mobile phase on the retention and selectivity of five common flavonols (Fig. 1). Zwitterionic stationary phase with polymeric skeleton was used in this study as that column can be used in basic conditions, which is not indicated for silica-based column. The advantage of application HILIC column for flavonols separation should be shortening of analysis time due to their low hydrophilic properties.

2. EXPERIMENTAL

2.1. Reagents

The commercial standards of flavonoids as well as the other chemicals were purchased from Sigma (Steinheim, Germany) or from Extrasynthesis. Methanol and acetonitrile were of HPLC grade from Merck (Darmstadt, Germany). Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) with the electrical resistivity of 18 MΩ×cm was used in all experiments. Stock solutions of flavonoids as well as their diluted mixtures were prepared in acetonitrile. All solutions were filtered through PTFE 0.45 μm membrane filters (Millipore) and degassed prior to use.

2.2. Instrumentation

Chromatographic analysis was performed with the Shimadzu LC system consisted of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC, detector UV SPD 20A connected to 3200 QTRAP Mass spectrometer (Applied Biosystem/MDS SCIEX) via additional Valco valve. A MS system was equipped with electrospray ionization source (ESI) operated in negative-ion or in positive mode. ESI conditions were following: capillary temperature 450°C, curtain gas at 0.3 MPa, auxiliary gas at 0.3 MPa, ionisation mode source voltage 4.5 kV. Nitrogen was used as curtain and auxiliary gas. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined in infusion mode (Table 1) [15]. Standard solutions were infused into the electrospray source via a 50 μm i.d. PEEK capillary employing a Harward Apparatus pump at 10 μL/min. Continuous mass spectra were obtained by scanning *m/z* from 50 to 650.

Compounds were separated on SeQuant™ Zic-*p*HILIC column (20 x 2.1 mm, 5 μm) from Merck (Darmstadt, Germany) at 30°C. 8 mM formic acid (pH 2.8) or ammonium acetate buffer (pH 7 or 9) was used as eluent A and acetonitrile or methanol as eluent B. The mobile phase was delivered at 0.2 mL/min in isocratic or gradient mode. The analytes were identified by comparing retention time and *m/z* values obtained by MS and MS^2 with the mass spectra. Quantification of compounds was done from the calibration curves obtained in selected reaction monitoring (SRM) mode.

Table 1. LC/MS/M characteristics of flavonols in the negative mode and polarity of the analytes.

Compound	Q1	Q3	DP (V)	CE (eV)	logP
Quercetin	301	151	70	30	0.380
Rutin	609	300	80	48	-2.345
Quercitrin	447	300	60	30	-0.595
Kaempferol	285	151	70	28	1.043
Rhamnetin	315	165	35	30	1.189

Q1 - molecular ion; Q3 - fragmentation ion; DP - declustering potential, CE - collision energy

3. RESULTS AND DISCUSSION

Mobile phase composition is the most important parameter in chromatographic separation. In HILIC mode the large amount of organic solvent is commonly applied which can benefit the compounds with low aqueous solubility and also can enhance MS detection. In typical HILIC mobile phase, acetonitrile is used as a weak eluent. The eluent power change in order acetonitrile < ethanol < isopropanol < methanol < water [14].

Flavonols have pKa values in the range of 7-11 [16], thus, they exist mainly as neutral compounds in pH below 6 and in anionic form above pKa. Fig. 2 presents the influence of pH and methanol (MeOH) concentration on the retention factors of tested flavonols. As one can see there are no significant changes of retention when pH was increased from 2.8 to 9. Dissociation of analytes above its pKa should decrease the retention time if some electrostatic interactions with stationary phase analytes occur. Likewise, the electrostatic interactions with the residue of sulfobetaine of stationary phase would be greater in the case of positively charged molecules. For tested compounds partition mechanism is more probable.

Much more important is concentration of methanol. Presented results showed that retention times are proportional to methanol content in the mobile phase and increase according to the polarity of the solutes. Table 1 presents the values of logP calculated by QSAR (Quantitative structure-activity relationship) model. Theoretical LogP values have been predicted using QikProp [17] as implemented in Maestro 9.2 software (Schrödinger, LLC, New York, NY, 2011).The more polar compounds have lower logP. Elution order of tested flavonols agrees with their polarity and is opposite to elution order obtained in reverse-phase mode separation [15]. Rutin, the most polar analyte, is strongly retained with all eluents.

Comparison of retention times for flavonols using different methanol or acetonitrile in the mobile phase at pH 7 is presented on Fig. 3. The effect of organic solvent methanol or acetonitrile (ACN) in isocratic conditions is presented on Fig. 3. The slightly decrease in the retention times for weakly retained compounds rhamnetin, kaempferol, quercetin was observed, when methanol was

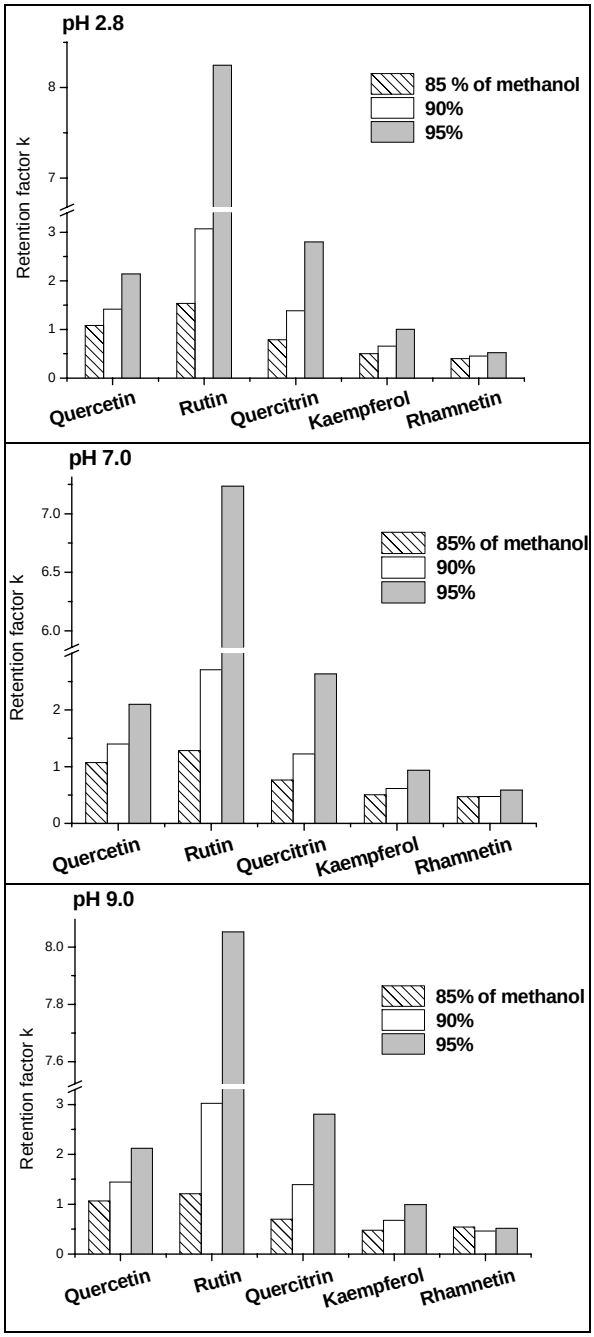

Fig. 2. Retention factor *k* as a function of methanol content and pH of the mobile phase. Column *p*ZIC-HILIC (20.2 x 2.1, 5 μm).

substituted by acetonitrile. MeOH can act as a donor of hydrogen bonds, whereas acetonitrile acts as an acceptor of hydrogen bonds and furthermore provides stronger dipole-dipole interactions. Liu *et al*. [14] suggested that methanol is able to

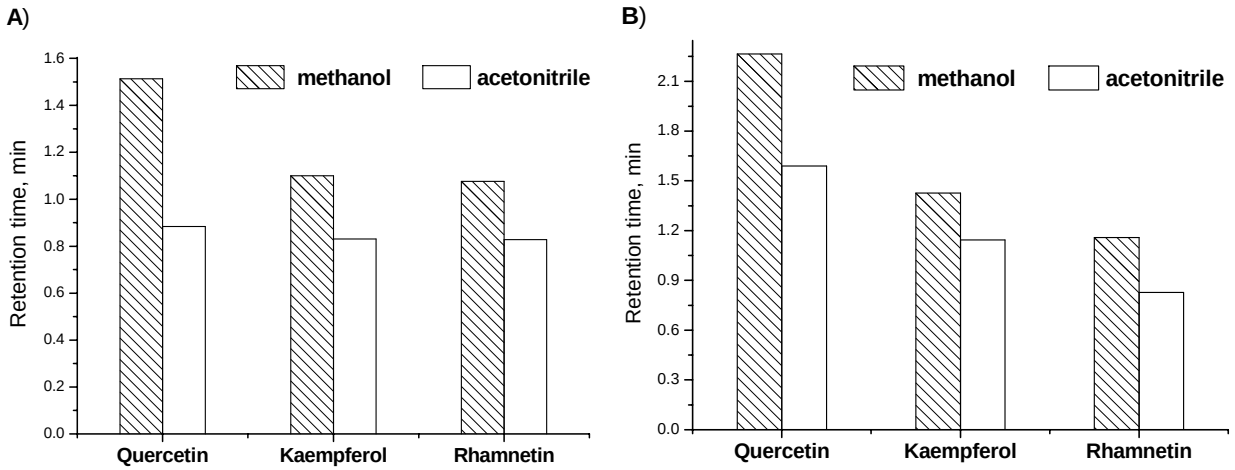

Fig. 3. The influence of content of methanol or acetonitrile in the mobile phase, pH 7; (A) 85%; (B) 95%. Column *p*ZIC-HILIC (20.2 x 2.1, 5 μm).

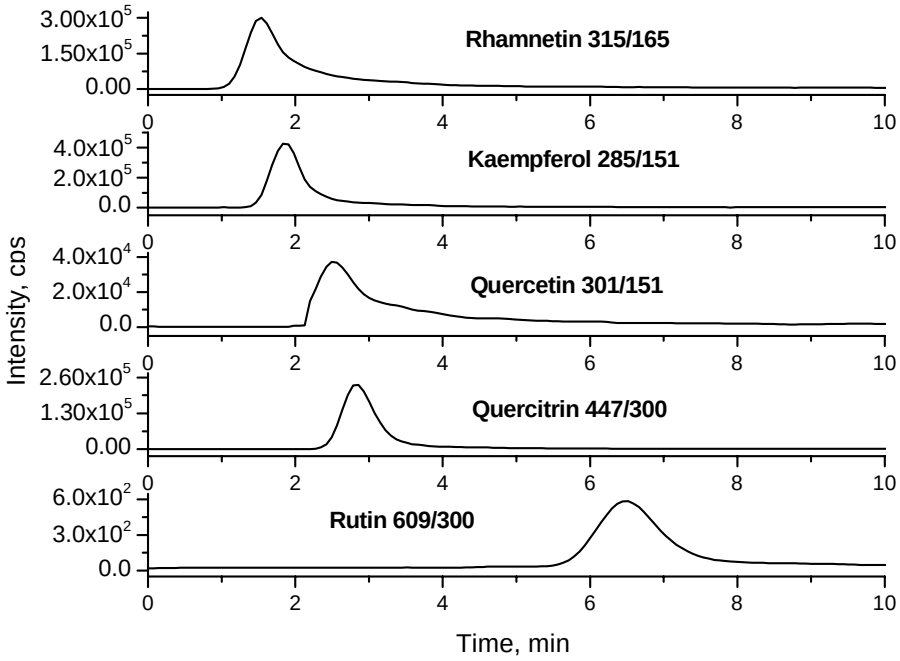

Fig. 4. Extracted ion chromatograms in SRM mode with eluent containing 95% of MeOH and buffer at pH 7.

compete for polar sites of the stationary phase disturbing the formation of aqueous layer required for partition mechanism. This leads to a more hydrophobic aqueous stationary phase and consequently in a poor retention of analytes with a high capacity to form hydrogen bonds [14, 18]. The obtained results show that for more polar compounds such as glycosides - rutin and quercitrin - methanol acts as a stronger solvent than ACN. The eluent with the same content of ACN instead of MeOH (95% or 85%) did not elute rutin or quercitrin from stationary phase within 120 minutes. Typical chromatogram obtained in isocratic condition for 95% MeOH is presented on Fig. 4.

Table 2. Retention time of flavonols at different ammonium acetate concentrations, pH 7.

Concentration (mM)	Rhamnetin	Kaempferol	Quercetin	Quercitrin	Rutin
0	1.27	1.59	2.35	2.81	6.57
10	1.40	1.69	2.50	2.87	6.29
25	1.46	1.64	2.44	2.69	5.85
50	1.46	1.75	2.69	2.58	5.67

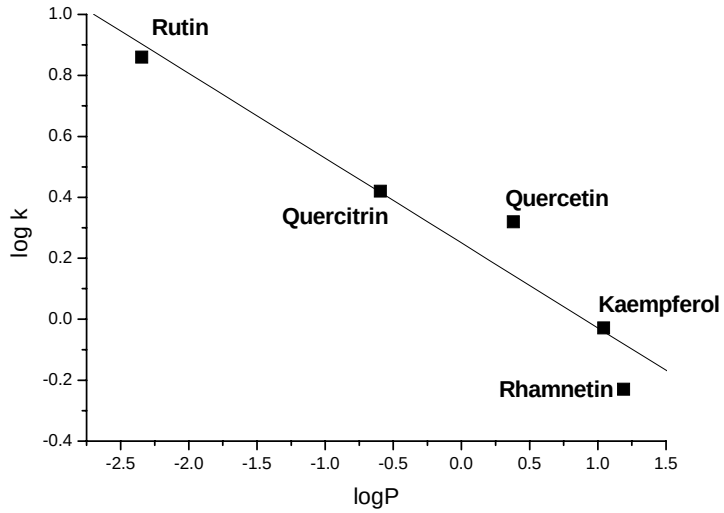

Fig. 5. Graphic correlation obtained between logP and log *k* for studied flavonols.

The peak shape obtained for rutin suggested mixed interaction mode.

Ionic strength can also affect the retention and selectivity of polar compounds in HILIC. The increase in salt concentration usually leads to an increase in the retention of analytes [19]. Table 2 presents the influence of ammonium acetate buffer concentration, pH 7, in range from 0 to 50 mM in the eluent. For more hydrophobic aglycons such as rhamnetin, kaempferol and quercetin, the small increase in retention times was observed. This indicated that the electrostatic repulsion was probably not the only factor that caused the increase in retention. In addition, it indicated that increasing salt concentration resulted in a smaller, but significant decrease in the retention time of rutin. Higher salt concentrations increased the eluting strength of the mobile phase, thus leading to decreasing retention.

Since hydrophilic interaction is one of the mechanisms that govern retention in HILIC mode, the hydrophobic properties of a compound should at least partly determine its behavior during separation. LogP is a measure of hydrophilic character of analyte: high logP values are measured for compounds with low hydrophilic character. In HILIC, analytes were postulated to partition between organic rich mobile phase and the water rich pseudostationary phase that is immobilized on the stationary phase. Thus the more hydrophilic compound (much polar) has the longer retention time and lower logP. As it is shown in Fig. 5, the relationship between log k and logP values for five flavonols under study is inversely correlated with R^2=0.9685. These results suggest that hydrophilic partition interaction was only one of the mechanisms involved in retention in the HILIC mode. Other interactions like hydrogen bonding, dipole-dipole should be taken

also into account. The differences between quercetin and rhamnetin suggested that existence of metoxy group instead of hydroxy is responsible for higher hydrophobic character of rhamnetin.

ACKNOWLEDGMENTS

The authors would like to thank the Structural Research Laboratory (SRL) at the Department of Chemistry of University of Warsaw for using HPLC-MS. SRL has been established with financial support from European Regional Development Found in the Sectorial Operational Programme “Improvement of the competitiveness of Enterprises, years 2004-2005” project no: WPK_1/ 1.4.3./1/2004/72/72/165/2005/U.

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