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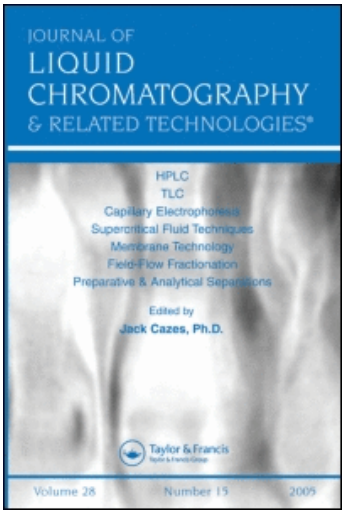

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Retention and Separation Selectivity of Natural Phenolic Antioxidants on Zirconia Based Stationary Phases

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Abstract: Many columns for reversed phase HPLC, based on silica gel, frequently used for separation of natural antioxidants, have a limited thermal and pH stability. In contrast, zirconium dioxide based stationary phases have an extremely different surface chemistry and are chemically stable over a wide rage of pH (1 to 14). Chromatographic properties associated with attractive and repulsive ionic interactions were investigated by measuring the retention data of isomeric naphthalene disulphonic acids on three zirconia based columns with carbon, carbon C_{18}, and polystyrene bonded stationary phases. A comparative study on the retention behavior of natural antioxidants on zirconia based stationary phases is reported. The results suggest that zirconia based columns could be successfully used for separation of natural phenolic antioxidants.

Keywords: Zirconia carbon, Zirconia carbon C_{18}, Zirconia polystyrene, Bonded phases, Natural antioxidants, Phenolic compounds

INTRODUCTION

Several natural compounds (phenolic compounds, benzopyran hydroxylated derivates) that contain one or more hydroxyl groups bonded by a benzene

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ring are known as natural antioxidants. These compounds can prevent the oxidative process and can play an important role in human health, having antimutagenic and antitumoral properties.[1,2]

The most commonly used analytical techniques for natural antioxidants include spectrophotometry,[3] capillary electrophoresis,[3–5] thin layer chromatography, gas chromatography, and most frequently, high performance liquid chromatography.[6–15] Hyphenated techniques, such as HPLC-MS[6,7] or HPLC-NMR,[16] and recently, two dimensional liquid chromatography[17,18] play an increasing role in the analysis of natural antioxidants. Octyl, octadecyl, phenyl, polyethylene glycol, pentafluorophenylpropyl, etc., stationary phases chemically bonded on a silica gel support, are used most frequently in the HPLC separation of natural phenolic antioxidants.[4–15]

Because some of these compounds such as polyphenolic carboxylic acids are strongly polar, acidic mobile phases in the pH range between 2.4 and 4.0 are recommended to suppress the dissociation of weak acids. Silica gel based stationary phases usually are not stable enough in either acidic or basic media, where either hydrolysis of the bonded ligands or slow dissociation of the matrix may occur, resulting in bleeding of the stationary phase, gradual decrease in the retention, and poor peak symmetry.

Zirconia dioxide stationary phase support is chemicaly stable over a wide pH range (from 1 to 14)[19,20] and has excellent thermal stability.[21,22] This phase can be coated with polybutadiene, polystyrene, or with a thin film of pyrolytic carbon, by heating it to 700–800°C.[23] Carbon clad zirconia can be modified by covalently bonding octadecyl groups, preserving the excellent thermal and pH stability of the zirconia suport. The goal of this work is to compare the separation selectivity of three zirconia based stationary phases for natural phenolic antioxidants. The chromatographic applications of zirconia based phases were previously studied for various compounds.[24–30] The Zirconia Carbon column provides interesting differences in separation selectivity from alkylsilica gel columns, useful for two-dimensional separations of phenolic antioxidants.[17] However, chromatographic behaviour of these compounds on various types of zirconia phases has not been systematically compared so far, except for the separation of oxyethylene glycol and oxypropylene glycol surfactants.[37]

EXPERIMENTAL

Materials and Reagents

For all measurements, methanol of HPLC grade (Lichrosolv, Merck, Darmstadt, Germany) and distilled water, purified using a Milli-Q water purification equipment (Millipore Intertech, Bedford, MA, USA), were mixed to

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Table 1. Phenolic antioxidants tested

No.	Compounds	Manufaturer
1	4-Hydroxybenzoic acid	Sigma Aldrich
2	Protocatechuic acid, 3,4-dihydroxybenzoic acid	Sigma Aldrich
3	Gallic acid, 3,4,5-trihydroxybenzoic acid	Sigma Aldrich
4	4-Hydroxy-phenylacetic acid	Sigma Aldrich
5	Vanillic acid, 4-hydroxy-3-methoxybenzoic acid	Fluka
6	Caffeic acid, 3,4-dihydroxy-cinnamic acid	Sigma Aldrich
7	Catechin, *trans*-3,3′,4′,5,7-pentahydroxyflavane	Fluka
8	Chlorogenic acid, 3-0-(3,4-dihydroxycinnamoyl)-D-quinic acid	Fluka
9	4-Hydroxycoumarin, 4-hydroxy-1-benzopyran-2-one	Sigma Aldrich
10	Syringic acid, 3,5-dimethoxy-4-hydroxybenzoic acid	Sigma Aldrich
11	Ferulic acid, 3-(4-hydroxy-3-methoxyphenyl)-propenic acid	Sigma Aldrich

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prepare the mobile phases. Sodium sulphate and phosphoric acid (all of reagent grade) were obtained from Lachema (Brno, Czech Republic). All solvents were filtered using a Millipore 0.45 μm filter and degassed in an ultrasonic bath before use.

The naphthalene sulphonic acid standards were obtained from Synthesia (Semtin, Czech Republic). Benzene, ethyl benzene, toluene, aniline, phenol, and the standards of phenolic compounds and flavonoids (natural antioxidants) listed in Table 1 were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Fluka (Buchs, Switzerland).

Equipment

A HP 1090 liquid chromatograph equipped with a UV diode array detector, an autosampler injector, and a Series 7994A workstation (Hewlett-Packard, Palo Alto, CA, USA) were used for measurements.

For the chromatography of naphthalene disulphonic acids (NDSA), an isocratic instrument including a chromatograph LC-10AD pump (Shimadzu, Kyoto, Japan) and a variable-wavelength UV detector (LCP 2564, ECOM, Prague, Czech Republic) operated at 254 nm were used. A personal computer with a CSW chromatographic data station was employed to collect the detector data. Sample injection was performed using a Rheodyne model 7125 sampling valve with a 20 μL sample loop (Berkeley, CA, USA). The columns used in this work and their characteristics are listed in Table 2.

Table 2. Characteristics of the tested zirconia-based chromatographic columns

Column name, dimensions, length × inner diameter (mm), particle size	Support and bonded phase	Specific surface (m^2/g)	V_M (mL)
Discovery ZR-Carbon 150 × 4,6; 5 μm	Zirconium oxide Carbon	30	1.78
Discovery ZR-Carbon C18 150 × 4,6; 5 μm	Zirconium oxide Carbon-octadecyl	30	1.58
Discovery ZR-PS 150 × 4,6; 5 μm	Zirconium oxide Polystyrene	30	1.51

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RESULTS AND DISCUSSION

Surface Properties of the Zirconia Based Stationary Phases

The surface chemistry of zirconia based stationary phases is extremely different from silica-gel phases. There are no silanol groups on the surface of zirconia dioxide, but hard Lewis acid sites with a strong affinity for Lewis bases (such as phosphate, fluoride, acetate, citrate, and carboxylate and anions). Both adsorption partitioning interactions may be associated with zirconia particles.[22] Our earlier study[36] showed that the results of the Galushko and Engelhardt tests[31–34] indicate a hydrophobicity of zirconium oxide Carbon C_{18} columns comparable with octadecylsilica columns. In this work, positive results of the conventional "silanol activity" tests were found for the zirconium oxide based chemically bonded stationary phases, which of course cannot be attributed to silanophilic interactions, but to other polar interactions on the stationary phase surface such as π-π electron interactions in the case of Zr-Carbon and Zr-Carbon-C_{18} columns.

To determine the ionic interactions of stationary phases, we measured the retention of naphthalene-disulphonic acids (NDSA) in purely aqueous mobile phases. The retention of NDSA isomers grows proportionally with the increase of the dipole moment, in the order 1,5 < 1,6 < 1,3 < 1,7 NDSA.[35]

While ionic exclusion of the naphthalene disulphonic acids, attributed to the Donnan effect repulsive type interactions of these compounds with ionized silanol groups was observed with silica gel based columns,[35] zirconium dioxide based columns contain no silanol groups and the retention of the naphthalene disulphonic acids on these columns is probably due to the π-π interactions of the planar naphthalene ring with the carbon layer stationary phase clad onto the zirconia support. The retention parameters of naphthalene-disulphonic acids on zirconia based columns are listed in Table 3.

Both the Zr-Carbon phase and the Zr-Carbon C_{18} phase retain strongly naphthalene disulphonic acids, which cannot be eluted under the test

Table 3. Retention data of NDSA, mobile phase 0.4 M Na_2SO_4, F = 1 ml/min, Tc = 30°C

Compound	Symbol	μ	Log (μ)
1,3 naphthalene disulfonic acid	1,3-NDSA	0.162	−0.79
1,5 naphthalene disulfonic acid	1,5-NDSA	0.000	—
1,6 naphthalene disulfonic acid	1,6-NDSA	0.139	−0.85
1,7 naphthalene disulfonic acid	1,7-NDSA	0.198	−0.70
		Discovery	
Column	ZR-Carbon	ZR-CarbonC18	ZR-PS
V_M	1,78	1,67	1,51
V_R			
1,5-NDSA	[a]	10.59	1.86
1,6-NDSA	[a]	59.23	2.58
1,3/NDSA	[a]	[a]	3.32
1,7/NDSA	[a]	[a]	4.04
F_{as} (NDSA)			
1,5	[a]	2.9	2.1
1,6	[a]	4.4	2.7
1,3	[a]	[a]	3.5
1,7	[a]	[a]	5.0
α (NDSA)			
1,6/1,5	[a]	4.59	0.39
1,3/1,5	[a]	[a]	0.79
1,7/1,5	[a]	[a]	1.17
A	—	—	2.26 ± 0.58
B	—	—	3.08 ± 0.74
R^2	—	—	0.94

V_M-columns hold-up volume (ml), V_R-retention volume (ml), F_{as}-asymmetry factor, α-elution ratio [$\alpha = (V_{RJ}/V_{RI})$-1], μ-dipole moment. A, B-best-fit regression parameters of the correlation equation log α = A+B log μ,[36] R^2-correlation coefficient.
[a]No elution.

conditions. This behaviour can be probably explained by strong interactions between the naphthalene ring and the carbon layer surface. On the Zr-polystyrene stationary phase, the interactions are weaker, because this phase does not contain a carbon layer deposited on the zirconia suport, so that the naphthalene-disulphonic acids can be eluted earlier (in 4 min) and all isomers are well separated under the test conditions (Figure 1).

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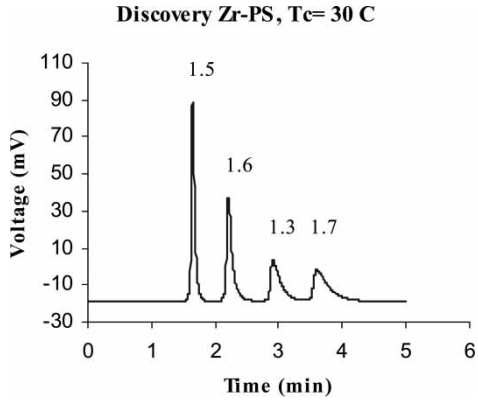

Figure 1. The separation of isomeric NDSA on Discovery Zr-polystyrene column, mobile phase 0.4 M Na_2SO_4 in water, Flow rate 1 mL/min, Tc = 30°C, UV detection, 254 nm.

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Hence, the retention of naphthalene-sulphonic acids on the Zr-polystyrene stationary phase results mainly from the solvophobic interactions with the polystyrene layer, much like as with the alkyl bonded silica gel columns,[35] as the elution order of the isomeric NDSA is the same on the two types of columns.

Comparison of the Retention of Phenolic Antioxidants on Three Zirconia Based Stationary Phases

The investigation of the chromatographic behaviour of synthetic oxyethylene-oxypropylene block (co)polymers on zirconia based stationary phases[37] showed that the retention and the selectivity of separation depend not only on the type of the stationary phase, but are strongly affected by the composition of the mobile phase. Based on our earlier study,[17] using a Zirconia-Carbon column in the second dimension of a comprehensive 2-D LCxLC system for the separation of phenolic antioxidants, we compared the retention behaviour of natural antioxidants on three types of Zirconia based stationary phases (Zirconia-Carbon, Zirconia-Carbon C_{18}, Zirconia-polystyrene). For this purpose, we used mobile phases containing 50 mM phosphoric acid in methanol and 50 mM phosphoric acid in water.

On a Discovery Zr-Carbon column with a thin layer of graphitized carbon deposited on the zirconia support, some natural antioxidants, mainly flavones, are strongly retained even in acidified non-aqueous organic mobile phases. However, the retention times of phenolic acids are long and the selectivity of separation is poor in methanol with phosphoric acid additive (Figure 2A). Without the addition of phosphoric acid, the phenolic acids are

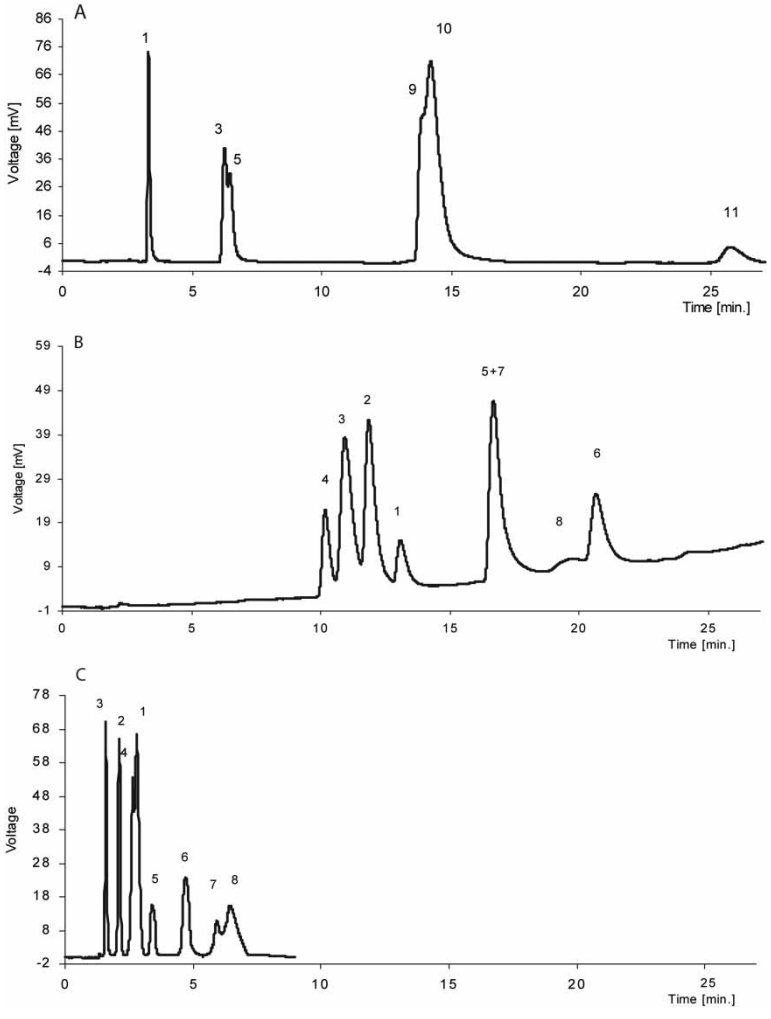

Figure 2. Separation of phenolic acids on three Zirconia columns. A) Discovery Zr-Carbon, mobile phase 50 mM H_3PO_4 in methanol, Flow-rate 1 mL/min., 40°C, B) Discovery Zr-Carbon C_{18}, mobile phase: 30-min linear gradient from 10 to 90% methanol at a constant concentration of 50 mM H_3PO_4, Flow-rate 1 mL/min., 40°C, gradient elution, C) Discovery Zr-PS, Mobile phase 50 mM H_3PO_4 in water, Flow-rate 1 mL/min., 40°C, Numbers of compounds are as in Table 1.

strongly retained in pure methanol and their peaks are badly tailing. The retention factors (k) of most phenolic acids increase approximately three times in the mobile phase containing 50 mM phosphoric acid in methanol: water 70:30 v/v (Table 4).

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Table 4. Retention times, tr, and retention factors, k, of phenolic acids

No.	Analyzed compounds	tr_A (min)	k_A	tr_B (min)	k_B
1	4-Hydroxy benzoic acid	6.24	2.54	3.31	0.85
3	Gallic acid	8.79	3.96	6.17	2.46
5	Vanillic acid	13.81	6.81	6.20	2.47
9	4-Hydroxy coumarin	31.29	16.7	13.96	6.83
10	Syringic acid	38.58	20.7	14.34	7.06
11	Ferulic acid	80.43	44.6	26.45	13.82

Zr-Carbon column with two different mobile phases: A-50 mM H_3PO_4 in methanol: water (70:30 v/v), and B-50 mM H_3PO_4 in methanol. Flow rate 1 ml/min, Tc = 40°C, isocratic elutions.

On a Discovery Zr-Carbon C_{18} column, the retention can be attributed mainly to the reversed phase mechanism, combined with the adsorption on the rigid carbon surface.[21] The chromatographic behaviour of natural antioxidants on a Discovery Zr-Carbon C_{18} column is characterized by the absence of retention in the acidic non-aqueous organic mobile phases and a very strong

Table 5. Retention times, tr, and retention factors, $k = (t_R/t_M) - 1$ for natural antioxidants on three zirconia-based stationary phases. Mobile phase: A) 50 mM H_3PO_4 in methanol, isocratic elution; B) 30-min linear gradient from 10 to 90% methanol at a constant concentration of 50 mM phosphoric acid, C) 50 mM H_3PO_4 in water, isocratic elution.

		Zr-Carbon (A)		Zr-Carbon-C_{18} (B)	Zr-PS (C)	
No.	Compounds	t_R (min)	k	t_R (min)	t_R (min)	k
1	p-Hydroxybenzoic acid	3.31	0.85	12.9	2.87	1.06
2	Protocatechuic acid	[a]	[a]	11.64	2.31	0.66
3	Gallic acid	6.17	2.46	10.85	1.60	0.36
4	4-Hydroxy-phenylacetic acid	[a]	[a]	10.06	2.65	0.76
5	Vanillic acid	6.20	2.47	16.47	3.44	1.75
6	Caffeic acid	[a]	[a]	20.25	4.74	2.41
7	Catechin	[a]	[a]	16.83	5.99	2.81
8	Chlorogenic acid	[a]	[a]	18.96	6.49	5.10
9	4-Hydroxy coumarin	13.96	6.83	[a]	[a]	[a]
10	Syringic acid	14.34	7.06	[a]	[a]	[a]
11	Ferulic acid	26.45	13.8	[a]	[a]	[a]

[a]Not tested.

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retention in water with phosphoric acid as the mobile phase, which is characteristic for the reversed phase retention mechanism attributed to the bonded octadecyl groups. The best separation on this column is obtained using gradient elution with a 30 min linear gradient from 10 to 90% methanol at a constant concentration of 50 mM phosphoric acid, see Figure 2B.

The retention of natural phenolic antioxidants on a Discovery Zr-PS column (zirconia particles coated with polystyrene) is less strong than on a Zr-Carbon C_{18} column and partial separation of eight sample compounds can be achieved in approximately eight minutes, using 50 mmol/L H_3PO_4 in water as the mobile phase (Figure 2C). This chromatographic behaviour might be due to less strong interactions of the stationary phase with natural phenolic antioxidants in comparison to the Zr-Carbon C_{18} column. All the three zirconia based columns studied show significant differences in the retention and in the separation selectivity.

The lowest values of the retention factors among all three zirconia columns (Table 5) were recorded with the zirconia-polystyrene column in aqueous phosphoric acid as the mobile phase. Higher retention was observed with the zirconia-carbon phase with acidified aqueous-organic mobile phases. Taking into account the peak shape, time, and the resolution as the criteria of the quality of separation, the zirconia-polystyrene stationary phase provides the best separation of natural phenolic antioxidants from among all the tested zirconia based phases and the shortest separation time (Figure 2).

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CONCLUSIONS

The results of the present study show that combined hydrophobic, polar, and electrostatic interactions strongly affect the chromatographic retention on zirconia based stationary phases and cause significantly different retention and separation selectivity for various types of zirconia columns. The retention of phenolic acids on the Discovery Zr-Carbon column is strong not only in aqueous-organic mobile phases, but also in methanol with the addition of phosphoric acid.

The octadecyl bonded groups of the Discovery Zr-Carbon-C_{18} stationary phase show non-polar interactions and a decrease of the interactions between the aromatic elements in the structure of natural antioxidants on one side and the carbon deposited on the zirconia support on the other side. Gradient elution significantly speeds up and improves the separation.

The Discovery Zr-PS column provides good selectivity for hydrophobic compounds. The separation of the phenolic compounds is relatively fast, even when using purely aqueous mobile phases with phosphoric acid. The selectivity of separation and the peak shapes are better and the running time is shorter than with other zirconia based columns. Due to these practical

advantages, the zirconia-polystyrene column can be recommended for routine analyses of natural phenolic antioxidants.

Because of a high chemical and thermal stability, the Discovery Zr-Carbon, Discovery Zr-Carbon C_{18}, and Discovery Zr-PS columns offer an efficient alternative to the traditional silica gel based columns for the separation of phenolic natural antioxidants. Unfortunately, most flavone antioxidants are too strongly retained on zirconia based columns.

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