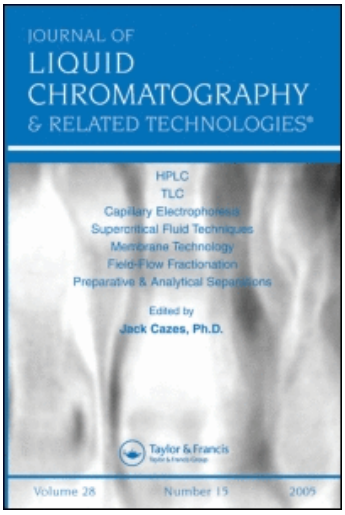

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Selectivity of a Native β-Cyclodextrin Column in the Separation of Catechins

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Selectivity of a Native β-Cyclodextrin Column in the Separation of Catechins

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Abstract: Catechins are most commonly analyzed by liquid chromatography using a C_{18} column and reversed mobile phases, involving hydrophobic interactions. It is showed that a β-cyclodextrin (β-CD) column can separate five catechins (catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate) as well as gallic acid in the reversed phase mode with methanol-water mobile phases and in the polar organic mode with acetonitrile-methanol mobile phases. 0.1% v/v of acetic acid had to be added to all mobile phases to improve peak shapes. The selectivity (order of retention of the catechins) is completely different between classical C_{18} columns and the β-CD column. This is due to a classical hydrophobic mechanism on C_{18} column compared to a H-bond dominated mechanism with β-CD column in both RPLC and polar organic mode. It is shown that the catechin solutes could be analyzed ten times faster using the low viscosity polar organic mode and a β-CD column compared to RPLC with a C_{18} column.

Keywords: Cyclodextrin stationary phase, Catechins of tea, Polar organic mobile phases, Reversed phase liquid chromatography

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INTRODUCTION

Flavonoids are polyphenols commonly found in the vegetal world. These compounds are abundantly present in the human diets.[1] They are believed to have many benefits to human health. Non exhaustive examples include: 1) antioxidant activity, 2) antibacterial and antiviral activity, 3) protection against several forms of cancers, 4) anti-inflammatory effect and 5) stimulation of the immune system without inducing allergic reactions.[2] Flavonoids are based on a diphenylpropane skeleton.[1] The monomeric flavonoids are divided into five subclasses: anthocyanidins, catechins, flavanones, flavones and flavonols.[1]

Catechins are flavonoids with sp^3 hybridized carbon in the C3 and C4 position when all other flavonoids have a sp^2 hybridized carbon in C4. The C3 position is also sp^2 hybridized except for flavanones. The five most common catechins are (+)-catechin, (−)-epicatechin, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) (Fig. 1). If (+)-catechin and (−)-epicatechin are diastereoisomers, they are not mirror image one of the other (enantiomers). They usually do not need a chiral stationary phase (CSP) to be separated.

Cyclodextrins (CD) were used as soon as the early eighties as successful CSPs in gas chromatography as well as liquid chromatography.[3–8] α, β, and γ cyclodextrins are macrocyclic systems made respectively by six, seven or eight α-1 $\rightarrow$ 4-glycosidically connected glucose molecules. In liquid chromatography, the CD-bonded stationary phase can be used in the reversed phase mode (RPLC) with hydro-organic mobile phase as well as in the polar organic mode with 100% organic solvent mobile phases.[8]

Flavonoid aglycones are most often analyzed in LC using RPLC with hydro-alcoholic mobile phases and alkyl bonded stationary phases and especially octadecyl (C_{18}) bonded phases.[9–13] CD bonded stationary phases were found very useful in the analyses of polar molecules such as sugars,[14–16] so it seems that they could be a valuable alternative stationary phase in the LC analysis of polar flavonoid aglycones and especially, catechins.

EXPERIMENTAL SECTION

Chemicals

Methanol and acetonitrile were HPLC grade solvent supplied by Fisher, Fair Lawn, New Jersey, USA. Acetic acid was from EM Science, Gibbstown, NJ. The catechin standards were from Unilever Research Laboratory, Colworth, UK. Acetic acid and all other chemicals were from Sigma, St Louis, Missouri, USA. $\sim 1\,g\,L^{-1}$ stock solution of the catechin was prepared in methanol and stored in the dark at 4°C. Fresh working solutions were

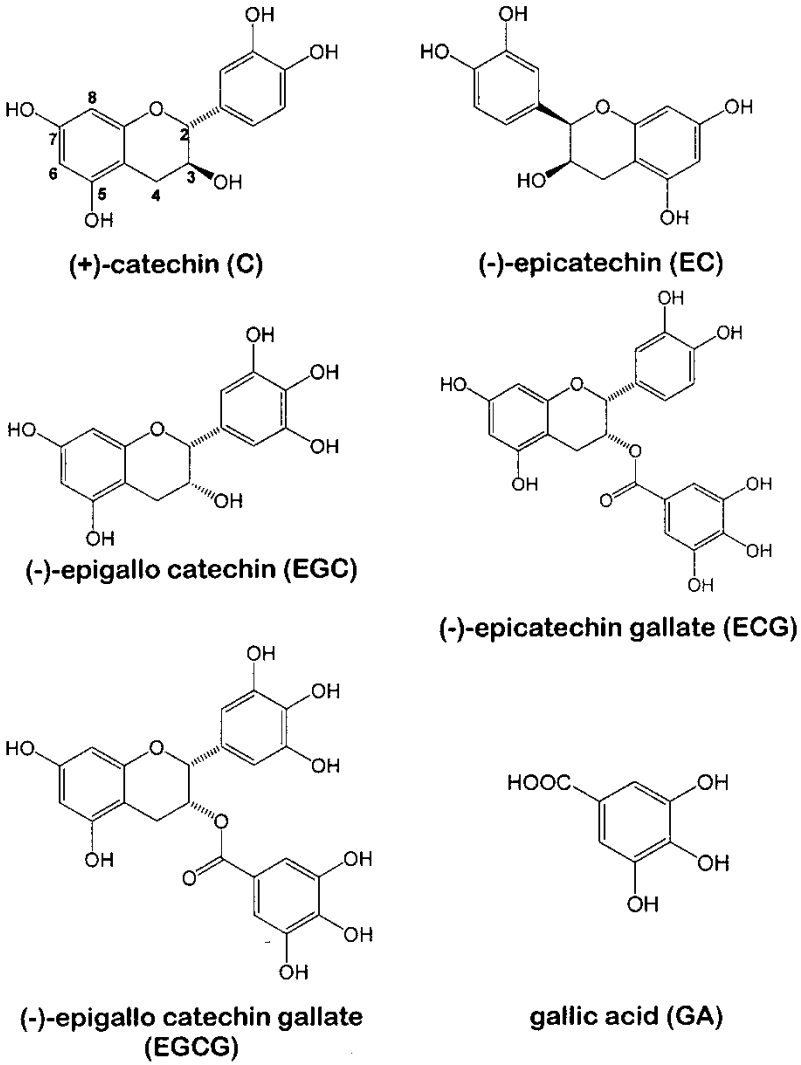

Figure 1. Chemical structures of the five catechins and gallic acid.

prepared daily, to avoid any oxidation, diluting the stock solutions with the working mobile phase.

Chromatography

A Shimadzu LC chromatograph was used with two LC10AD pumps, a SCL10 controller, a SPD6 UV detector and a CR3A integrator. The native β-CD

bonded columns were 25 cm, 4.6 mm i.d. Cyclobond I-2000 from Astec Whippany, New Jersey, USA. The column dead volume is a critical parameter used in all retention factor calculations. It was taken as the first detector signal change observed after an injection. It was always close to 3.2 mL.

RESULTS AND DISCUSSION

Polar Organic Mode

Since the β-CD bonded columns can work in polar organic mode with waterless mobile phases, this mode was studied first. The β-CD cavity can make inclusion complexation with solutes that would fit its size. In polar organic mode, there is no inclusion complexation. The apolar CD cavity is occupied by acetonitrile molecules in great excess compared to any injected solute. These solvent molecules are not displaced by solute molecules. The separation is due to polar and hydrogen bonding interaction between the solute hydroxyl groups and the CD hydroxyl group. Addition of trace amounts of acid and/or methanol allows adjusting for solute retention and selectivity.[8–10]

Effect of Mobile Phase Acid Content

Two compounds: gallic acid (GA) and epicatechin gallate (ECG) were selected to perform a study on the effect of small additions of acetic acid on solute retention and peak shape. Fig. 2 shows the effect of 0–0.5% v/v acetic acid added to two waterless polar organic mobile phases: pure acetonitrile and acetonitrile-methanol 90/10% v/v.

Below 0.1% v/v, the first trace amounts of acetic acid have a strong effect on both retention and peak shape. With the 100% acetonitrile mobile phase, retention factors are decreased by a factor ten and peak efficiency is doubled by the addition of 0.1% v/v acetic acid. The decreases in retention factor and peak shape improvements are somewhat lower with the acetonitrile-methanol 90/10% v/v mobile phase. The changes in both retention factor and peak shape are less dramatic when more acetic acid is added to the mobile phase. The 0.1% v/v acetic acid content will be used in all mobile phases for the rest of the study.

These results evidence the key role of hydrogen bonding in both the thermodynamic (retention factors) and kinetic (peak efficiency) process of the solute-CD interaction. Acetonitrile is a polar aprotic solvent unable to make hydrogen bonds. In pure acetonitrile, there are strong hydrogen bonds between the hydroxyl groups of the CD moieties and the phenol groups of the solutes. These strong interactions produce high retention factors and broad peaks.

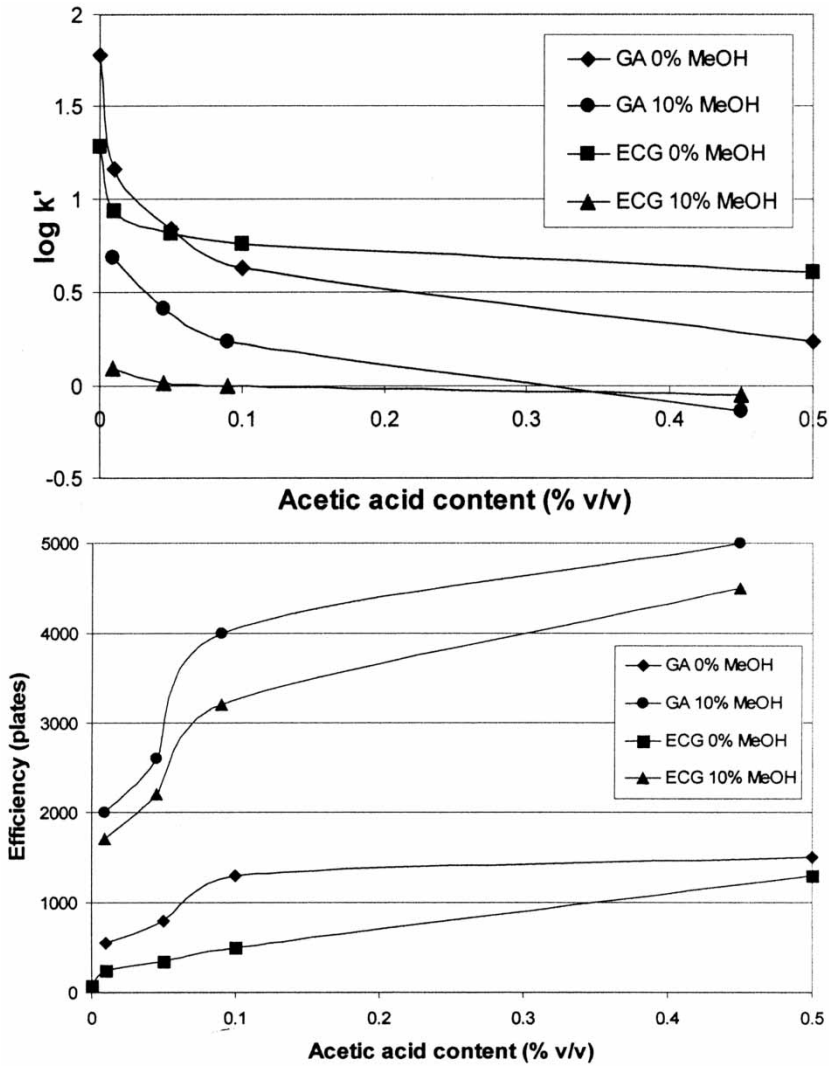

Figure 2. Effect of the acetic acid concentration in polar organic mode. Top: effect on the gallic acid (GA) and epicathechin gallate (ECG) retention factors. Bottom: effect on the GA and ECG peak efficiencies. Phases mobiles: 100% acetonitrile or acetonitrile/methanol 90/10% v/v.

Effect of Methanol Content

Acetic acid as well as methanol are competing with the solutes for H-bonds with the hydroxyl groups of the bonded CDs. They modulate the strength of the stationary phase-solute H-bond interactions. The effect of methanol on

solute retention was investigated with polar organic mobile phases all containing 0.1% v/v acetic acid. Fig. 3 shows the log k′ retention factor of the studied solutes plotted versus the mobile phase methanol content. The two isomers (+)-catechin and (−)-epicatechin (Fig. 1) both eluted very rapidly without being separated. The order of retention for the catechins is (+)-catechin and (−) epicatechin, EGC, ECG and EGCG. This order is not modified when the methanol content in the polar organic mobile phase changes (Fig. 3).

The retention order of gallic acid depends on the methanol content. For low methanol content (%MeOH $< 2\%$ v/v), gallic acid is eluted third, in between EGC and ECG. It is fourth for methanol content between 2 and 7% v/v eluting after ECG and befor EGCG. For methanol content higher than 7% v/v, gallic acid is the last eluting compound of the set. Gallic acid is not a member of the catechin family; its retention behavior differs from that of the catechins (Fig. 3). The effect of methanol on efficiency was not as significant as that of acetic acid as shown by Fig. 2 for 0% and 10% v/v methanol.

Reversed Phase Mode

RPLC with methanol-water mobile phase is the most common mode used for catechin separation with C_{18} columns.[9−13] The retention factors of the six catechins were studied in the full range of possible RPLC mobile phase compositions from 100% water to 100% methanol, all mobile phase containing 0.1% v/v acetic acid (1 mL L^{-1} or 1.05 g L^{-1} or 17.5 mM, aqueous pH = 3.2).

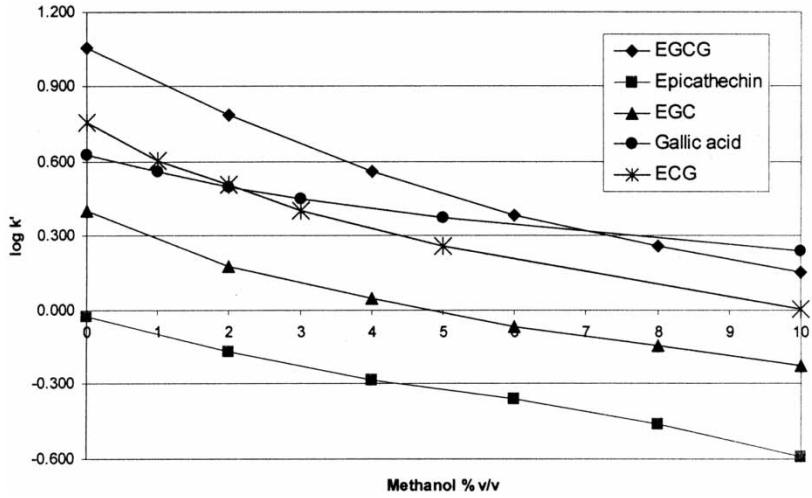

Figure 3. Effect of the polar organic mobile phase methanol content on catechins' retention factors. All mobile phases contain acetonitrile and 0.1% v/v acetic acid.

Fig. 4 shows the catechins and gallic acid retention factor evolution with the methanol content in the mobile phase. It should be noted that the compounds were injected one by one to draw Fig. 4. Indeed, like already observed in the polar organic mode, the β-CD column could not separate the stereoisomers catechin and epicatechin. Also, epicatechin and EGC eluted very close one to the other as did ECG and EGCG (Fig. 4).

In water-rich mobile phases (methanol $< 50\%$ v/v), the elution order observed with the RPLC mode is very similar to that observed with the polar organic mode. The gallic acid solute shows retention order reversal similar to the one described in polar organic mode. This result is surprising since it is known that water is the solvent with the highest hydrogen bonding capability. It should screen all hydroxyl groups on the stationary phase as well as the solutes.

The classical RPLC hydrophobic behavior is observed: the solute retention factors decrease as the methanol content increase. This behavior is not observed for methanol-rich mobile phases. It may be due to reduced solubility of catechins in pure methanol mobile phase. It should be pointed out that the retention factors are very small with methanol rich mobile phase, i.e. the solutes elute close to the dead volume. A small error in the dead volume estimation leads to large error in k′ calculations.

The RPLC efficiency was low in the 1000–1500 plate region for a 25 cm column (height equivalent to a theoretical plate $\sim$170–250 μm). It could be improved by changing the acid concentration and/or nature or using a silanol binding additive such as triethylamine.

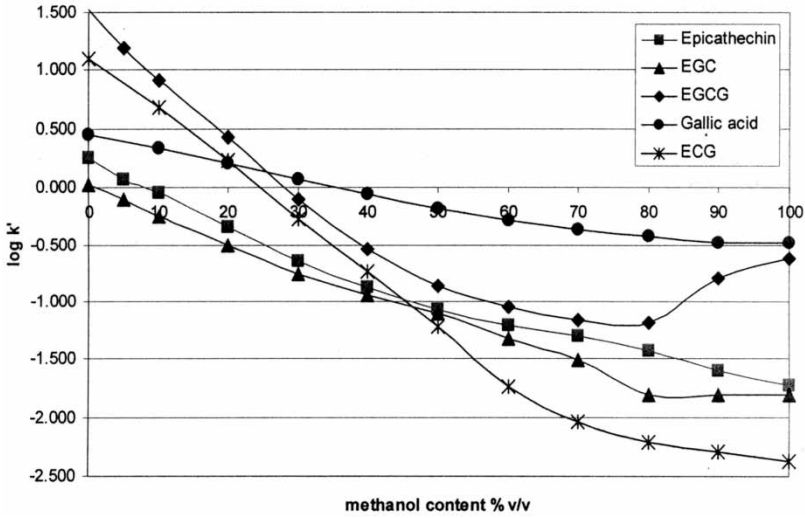

Figure 4. Effect of mobile phase methanol content on catechin retention factors in RPLC. All mobile phases are methanol-water mixture containing 0.1% v/v acetic acid.

Comparing Selectivity

The catechin retention order obtained with the β-CD column is similar in the two modes, RPLC and polar organic. However, it is very different from the retention order usually obtained in RPLC with classical C_{18} columns.[9–13]

Fig. 5 compare the separation of the same mixture of catechin standards on a classical C_{18} column (top chromatogram) in RPLC mode with a 80–20% v/v water/methanol mobile phase (+0.1% v/v acetic acid) and on the Cyclobond β-CD column (bottom chromatogram) with a polar organic

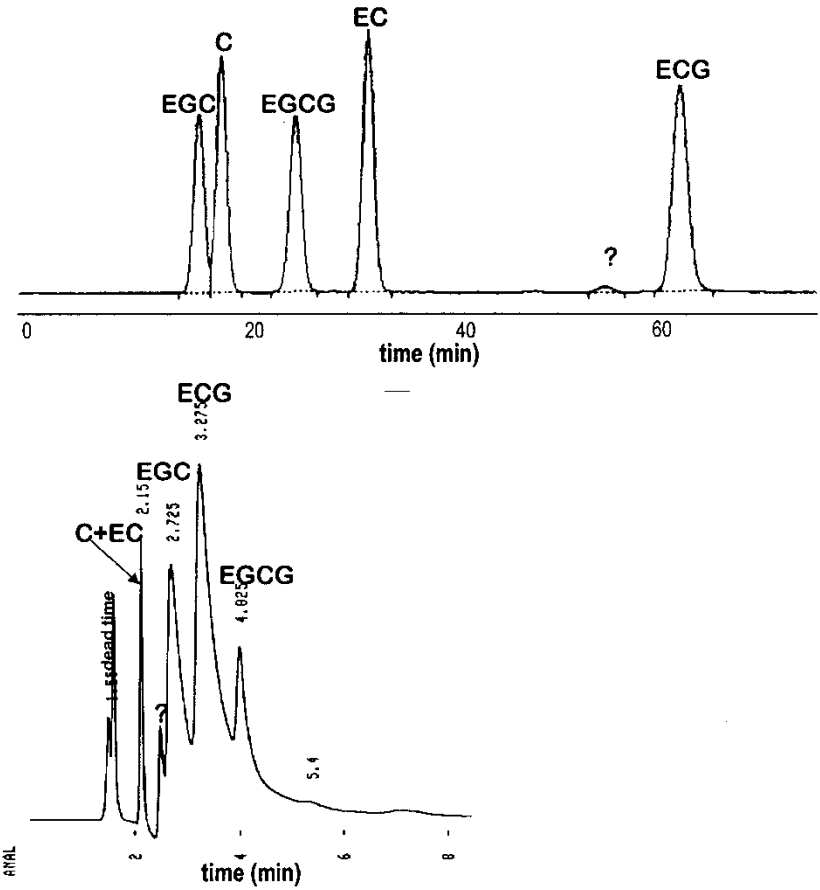

Figure 5. Catechin HPLC separation. Top: RPLC chromatogram, column Chrompack Inertsil ODS-2, 25 cm, 4.6 mm i.d., mobile phase: methanol/water 80/20% v/v, acetic acid 0.5% v/v, 0.5 mL min^{-1}, 40°C. Bottom: Polar organic separation, column Astec Cyclobond I-2000, 25 cm, 4.6 mm i.d., mobile phase: acetonitrile/methanol 94/6% v/v, acetic acid 0.1% v/v, 2 mL min^{-1}, room temperature.

Table 1. Chromatographic parameters of the C_{18} RPLC and β-CD polar organic Fig. 5 chromatograms and a β-CD chromatogram with a RP mobile phase

	20/80% v/v MeOH/water RPLC mobile phase						94/6%v/v ACN/MeOH		
	Column Inertsil C_{18}			Column Cyclobond I-2000 β-CD					
Solute	t (min)	k′	N (plates)	t[a] (min)	k′	N (plates)	t (min)	K	N (plates)
C	16.37	1.73	1,400	9.8	2.06	2,200	2.15	0.34	6,000
EC	28.25	3.71	2,400	4.58	0.43	1,600	2.15	0.34	6,000
EGC	14.55	1.43	1,200	4.19	0.31	1,600	2.725	0.70	400
ECG	63.35	9.56	5,500	12.7	2.97	600	3.275	1.05	300
EGCG	22.37	2.73	1,500	13.4	3.19	400	4.025	1.52	4,000

All mobile phases contain 0.1% v/v acetic acid.
[a]chromatogram not shown in Fig. 5.

mobile phase (acetonitrile/methanol 94–6% v/v + 0.1% acetic acid). Table 1 compares the chromatographic parameters obtained for these two chromatograms.

The ODS column separates all 5 solutes at baseline with a good efficiency at 0.5 mL min^{-1}. This good separation is at the cost of a lengthy experiment time. More than one hour is needed to elute ECG. A gradient elution would reduce the elution time but add re-equilibration delay between runs. The retention times obtained with the β-CD column are significantly lower than those obtained with the C_{18} column. This was observed in both RPLC and polar organic mode. The drawback is that, in RPLC, EC and EGC, and ECG and EGCG are four solutes eluting in only two peaks. The full selectivity study presented previously (Fig. 4) showed that the two co-elutions occur for any RPLC mobile phase composition. In polar organic mode, C and EC coelutes and the peak efficiencies for EGC and ECG are low, but the low mobile phase viscosity allows to use a high flow rate (2 mL min^{-1}) and the five compounds can be separated in less than 5 minutes. This saves 90% of the analysis time compared to the RPLC separation on a C_{18} column (Fig. 5 and Table 1).

Obviously, the selectivity obtained with the β-CD column (hydroxyl and H-bond interaction) differs completely from that obtain using any C_{18} column (hydrophobic interaction). For example, ECG is highly retained on C_{18} stationary phase due to its lower hydrophobicity compared to the other catechins. ECG has 7 hydroxyl groups in its molecule (Fig. 1) compared to 8 hydroxyl groups for EGCG that is more retained on the β-CD column. The unique selectivity of the β-CD column is mostly preserved in the RPLC mode (Table 1).

CONCLUSION

The β-CD column can provide selectivity toward the analysis of catechins that differ significantly from that obtained with classical C_{18} columns. The catechin elution order is mainly linked to the number of hydroxyl groups in the molecule. Using the polar organic mode to adjust the H-bond capabilities of the mobile phase, it is possible to modulate catechin retention and selectivity. Low amounts (few per thousand) of acetic acid are always needed to improve the peak shapes and separation efficiency. The use of β-CD columns could make catechins' analysis one order of magnitude faster than what is obtained with classical C_{18} columns.

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