Rapid Tea Analysis on Poroshell 120 SB-C18 with LC/MS

Application Note

Food and Beverage

Authors

Anne E. Mack and William J. Long
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

An analysis of ten compounds (9 catechins + caffeine) commonly found in green tea demonstrates similar selectivity on Agilent ZORBAX SB-C18 and Agilent Poroshell 120 SB-C18. The 1.4 min gradient analysis on Poroshell 120 generates linear calibration curves for all ten compounds through LC/MS. Several bottled and brewed tea samples are quantified and compared. An unfiltered, undiluted brewed green tea sample demonstrates a lifetime of more than 1500 injections on the Poroshell 120 column with a dirty sample at high pressure.

Introduction

Polyphenolic compounds reduce the risk of heart disease, prevent cancer, and combat other illnesses. A popular source of polyphenols is fresh tea leaves or green tea, which contain high levels of catechins. Catechins affect the color and flavor of tea, contributing to the characteristic bitterness associated with tea [1]. Of the catechins found in tea, epigallocatechin gallate (EGCG) is given attention, as it is the most abundant of the polyphenolic compounds found in tea extract [2].

While all teas originate from the same plant, Camellia sinensis, different processing methods produce varied teas. The amount of fermentation (oxidation) that a tea leaf undergoes following harvesting dictates what type of tea the leaf becomes. Tea leaves begin to wilt and oxidize quickly, if not dried soon after harvesting. During this process, the leaves darken as chlorophyll breaks down and tannins are released. The oxidation process is stopped at a controlled time by heating the leaves and deactivating the enzymes responsible for breaking down chlorophyll. Black tea is fully oxidized, oolong tea is semi-oxidized and green tea is un-oxidized [3,4]. Because oxidation lowers the catechin levels, green tea provides the highest quantity of catechin antioxidants per serving, while black tea delivers the least.

In this application note, an HPLC method for catechins in tea developed by Yoshida et al [5] on an Agilent ZORBAX SB-C18 column is transferred to a similar dimension Agilent Poroshell 120 SB-C18 column to demonstrate similar selectivity. The method is optimized for LC/MS. Calibrations curves are generated and tea samples, both bottled and brewed, are analyzed for comparison. Additionally, a lifetime study using an undiluted, unfiltered brewed green tea sample demonstrates the benefits of the Poroshell column's large 2-µm frits with dirty samples.

While a tea analysis via HPLC is not novel, this method shows Poroshell 120's effectiveness in analyzing other natural product samples. The Poroshell 120 column is shown to separate a group of 10 closely related compounds, including four epimer pairs using a representative sample that is affordable and easily obtainable.

Experimental

An Agilent 1200 Series Rapid Resolution LC (RRLC) system with an Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) was used for this work:

- G1312B Binary Pump SL with mobile phase A: various modifiers in H_2O (0.1% H_3PO_4, 0.2% HCOOH, 0.2% CH_3COOH, 0.02% CF_3COOH, 10 mM CH_3COONH_4 pH 3.6-5.6, and 10 mM $HCOONH_4$ pH 3-4.5), and B: CH_3CN. Gradient was 10% B at t_0, ramp to 15% B, and then ramp to 27% B. Gradient times vary depending on column dimensions and flow rate. See Table 1.
- G1367C Automatic Liquid Sampler (ALS) SL, injection volumes are dependent upon specific method parameters. See Table 1.
- G1316B Thermostated Column Compartment (TCC) SL with temperature controlled at 40 °C.
- G6410A QQQ Mass Spectrometer with MS Source: electrospray AP-ESI, drying gas temperature and flow: 350 °C, 10 L/min, nebulizer gas pressure: 50 psi, capillary voltage: ± 3500 V, in SIM mode, *m/z* values shown in Figure 1. Catechins are monitored in negative mode, while caffeine is monitored in positive mode.
- MassHunter versions B.02.01, B.02.00 and B.03.01 were used for data acquisition, qualitative and quantitative analyses respectively.

Table 1. Various Method Parameters for Catechin Analysis

	Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm (p/n 883975-902)	**Agilent ZORBAX RRHT SB-C18, 4.6 × 50 mm, 1.8 µm (p/n 827975-902)**	**Agilent Poroshell 120 SB-C18, 4.6 × 50 mm, 2.7 µm (p/n 689975-902)**	**Agilent ZORBAX RRHT SB-C18, 2.1 × 50 mm, 1.8 µm (p/n 827700-902)**	**Agilent Poroshell 120 SB-C18, 2.1 × 50 mm, 2.7 µm (p/n 689775-902)**	**Agilent Poroshell 120 SB-C18, 2.1 × 50 mm, 2.7 µm (p/n 689775-902)**	**Agilent Poroshell 120 SB-C18, 2.1 × 50 mm, 2.7 µm (p/n 689775-902)**	**Agilent Poroshell 120 SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)**
Flow rate (mL/min)	1.00	1.00	1.00	1.00	1.00	1.25	1.50	0.83
Mobile phase A	0.1% H_3PO_4 in H_2O	0.1% H_3PO_4 in H_2O	0.1% H_3PO_4 in H_2O	0.1% H_3PO_4 in H_2O	Various additives in H_2O	0.2% CH_3COOH in H_2O	0.2% CH_3COOH in H_2O	0.2% HCOOH in H_2O
Mobile phase B	CH_3CN	CH_3CN	CH_3CN	CH_3CN	CH_3CN	CH_3CN	CH_3CN	0.2% HCOOH in CH_3CN
10% B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15% B	7.50	2.50	2.50	0.50	0.50	0.42	0.36	1.25
27% B	15.00	5.00	5.00	1.00	1.00	0.83	0.71	2.50
Stop time (min)	15.00	5.00	5.00	1.40	1.40	1.20	0.95	4.00 (includes re-equilibration)
Post run time (min)	10.00	3.00	3.00	1.00	1.00	0.80	0.60	n/a
Overall cycle time (min)	25.00	8.00	8.00	2.40	2.40	2.00	1.55	4.00
TCC temperature (°C)	40	40	40	40	40	40	40	40
Injection volume (µL)	15.0	5.0	5.0	1.0	1.0 (LC/UV), 1.5 (LC/MS)	1.5	1.5	2.0
Sample concentration (mg/mL)	0.03	0.03	0.03	0.03	0.03 (LC/UV), 0.003 (LC/MS)	0.003	0.003	n/a
System pressure (bar)	84	169	117	575	380 (LC/UV), 425 (LC/MS)	505	585	540

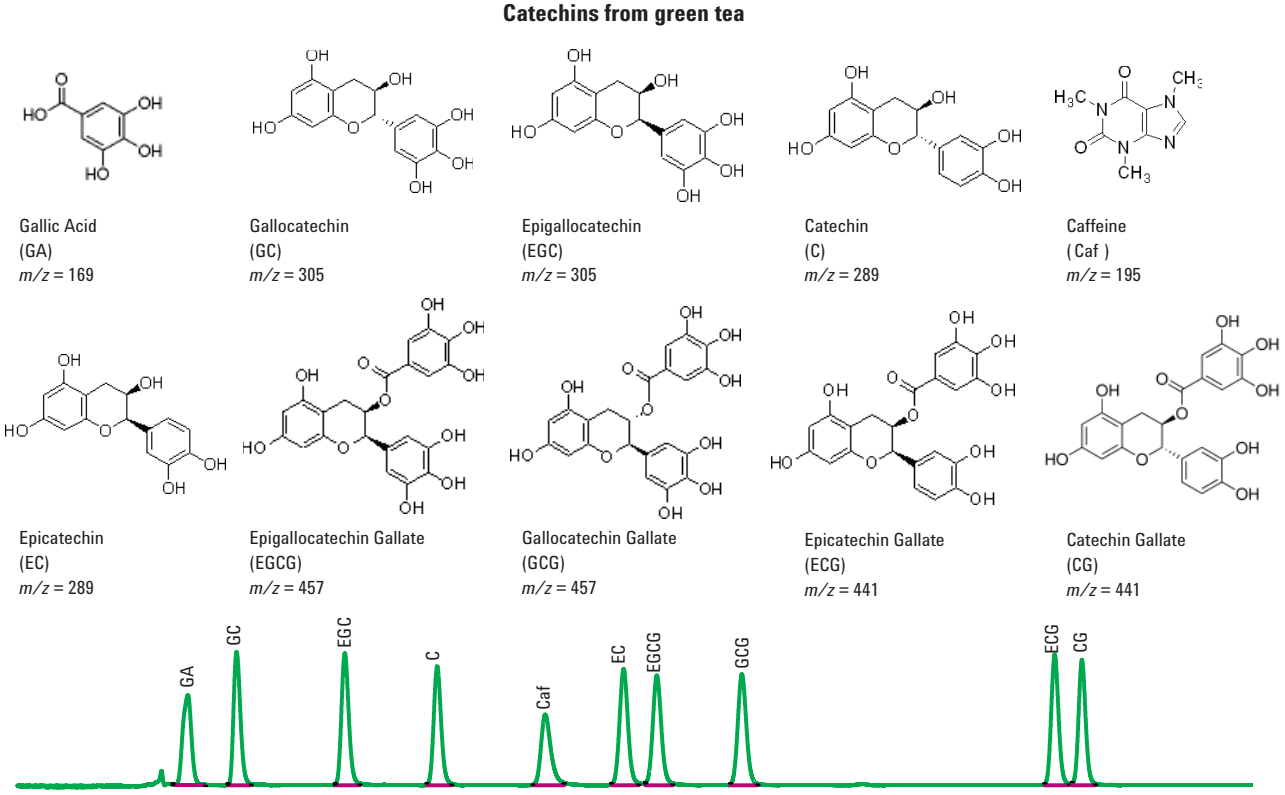


Figure 1. Compounds of interest, with elution order are shown on an Agilent ZORBAX SB-C18 with H_3PO_4 mobile phase. (Note: Selectivity may change slightly in subsequent chromatograms, but elution order remains constant.)

Six Agilent columns were used in this work:

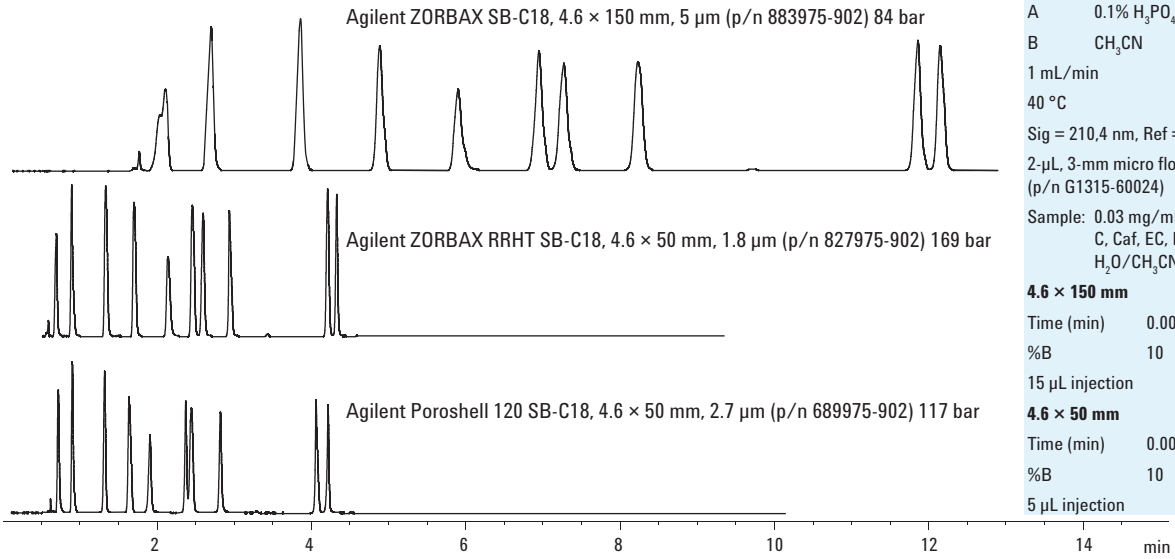
- Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm p/n 883975-902
- Agilent ZORBAX RRHT SB-C18, 4.6 × 50 mm, 1.8 µm p/n 827975-902
- Agilent Poroshell 120 SB-C18, 4.6 × 50 mm, 2.7 µm p/n 689975-902
- Agilent ZORBAX RRHT SB-C18, 2.1 × 50 mm, 1.8 µm p/n 827700-902
- Agilent Poroshell 120 SB-C18, 2.1 × 50 mm, 2.7 µm p/n 689775-902
- Agilent Poroshell 120 SB-C18, 2.1 × 100 mm, 2.7 µm p/n 685775-902

The compounds of interest are shown in Figure 1, with a chromatogram illustrating elution order. All analytes were purchased as dry powders from Sigma Aldrich (Bellefont, PA). Individual standards of gallic acid, epigallocatechin, catechin, caffeine, and epigallocatechin gallate were each prepared in H_2O at 1 mg/mL. Individual standards of gallocatechin, epicatechin, gallocatechin gallate, epicatechin gallate, and catechin gallate were each prepared in CH_3CN/H_2O (1:1) at 0.5 mg/mL. A composite sample was prepared by mixing 1 part each of the 1 mg/mL standards and 2 parts each of the 0.5 mg/mL standards, yielding 0.03 mg/mL of each analyte. Dilutions of this composite sample were prepared as necessary with H_2O. Tea samples were purchased locally, with the exception of bottled sample A, which was shipped from a colleague in Japan. Tea samples for quantitation (both bottled and brewed) were diluted 1:10 with H_2O prior to injection. The brewed green tea sample used for the lifetime study was not diluted or filtered prior to injection. Additionally, acetonitrile, phosphoric acid, formic acid, acetic acid, trifluoroacetic acid, ammonium acetate, and ammonium formate were also purchased from Sigma Aldrich (Bellefont, PA). Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Results and Discussion

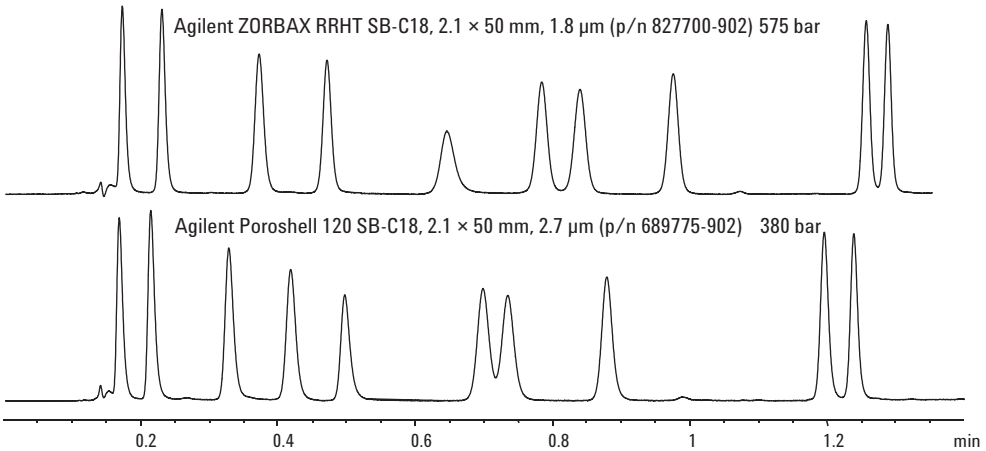
Previous work by T. Yoshida et al [5] shows a catechin analysis on an Agilent ZORBAX SB-C18, 4.6 × 150-mm, 5-µm column in 15 min scaled to an Agilent ZORBAX Rapid Resolution High Throughput SB-C18, 4.6 × 50-mm, 1.8-µm column in 5 min. Added to this work is an Agilent Poroshell 120 SB-C18 column for comparison. Figure 2 shows the time saved using shorter HPLC columns with smaller particle sizes, while maintaining resolution.

The method is scaled further to a 2.1 × 50 mm column in just over 1 min. The smaller column id allows the same analysis to run with lower flow rates, which are more suitable for MS work. The selectivity between the ZORBAX SB-C18 and Poroshell 120 SB-C18 is similar to allow for easy method transfer, as shown in Figure 3. System back pressure is a noticeable difference between the 1.8-µm ZORBAX column and the 2.7-µm Poroshell column. The larger superficially porous particles in the Poroshell column generate significantly less pressure than the smaller totally porous particles in the ZORBAX column. The Poroshell particles achieve similar performance due to a short mass transfer distance through the porous shell and substantially narrower particle size distribution as compared to the totally porous sub-2 µm material. In this case, the difference in pressure is enough to dictate whether a 400 or 600 bar instrument can be used.



A	0.1% H_3PO_4 in H_2O		
B	CH_3CN		
1 mL/min			
40 °C			
Sig = 210,4 nm, Ref = Off			
2-µL, 3-mm micro flow cell (p/n G1315-60024)			
Sample:	0.03 mg/mL each of GA, GC, EGC, C, Caf, EC, EGCG, GCG, ECG, CG in H_2O/CH_3CN		
4.6 × 150 mm			
Time (min)	0.00	7.50	15.00
%B	10	15	27
15 µL injection			
4.6 × 50 mm			
Time (min)	0.00	2.50	5.00
%B	10	15	27
5 µL injection			

Figure 2. Original 150-mm, 5-µm catechin method scaled to an Agilent ZORBAX SB-C18, 50-mm, 1.8-µm and to a 50-mm, superficially porous Agilent Poroshell 120 SB-C18, 2.7-µm.



A	0.1% H_3PO_4 in H_2O		
B	CH_3CN		
1 mL/min			
Time (min)	0.00	0.50	1.00
%B	10	15	27
40 °C			
Sig = 210,4 nm, Ref = Off			
2-µL, 3-mm micro flow cell (p/n G1315-60024)			
Sample:	1 µL injection of 0.03 mg/mL each of GA, GC, EGC, C, Caf, EC, EGCG, GCG, ECG, CG in H_2O/CH_3CN		

Figure 3. Catechin analysis is transferred to 2.1-mm id columns for use with LC/MS, analysis time is further reduced by maintaining 1 mL/min flow rate from original 4.6 × 150-mm method and scaling gradient times according to column volume.

The 2.1 mm id columns are suited for use with LC/MS due to the lower flow rates used. The original phosphoric acid mobile phase, however, is not compatible with the LC/MS system. Figure 4 shows several MS-friendly mobile phases that were screened for use with this catechin analysis. In addition to the results shown in Figure 4, a selection of 10 mM ammonium formate buffers were also screened from pH 3-4.5, the resulting chromatograms were nearly identical to the ammonium acetate data. Overall, the selectivity remained constant throughout this screening process. Consequently, the optimal mobile phase was selected based on signal strength of the analytes. All chromatograms in Figure 4 are shown on the same scale. Significant ion suppression is present with the buffers prepared from ammonium salts, as well as with the trifluoroacetic acid mobile phase. The two best contenders were formic acid and acetic acid, with acetic acid producing a slightly more intense signal for all compounds. It should be noted that the negative scans are shown as representative chromatograms in Figure 4, as the positive scans appeared to be less effected by ion suppression; however, the positive scans for caffeine still followed the same pattern.

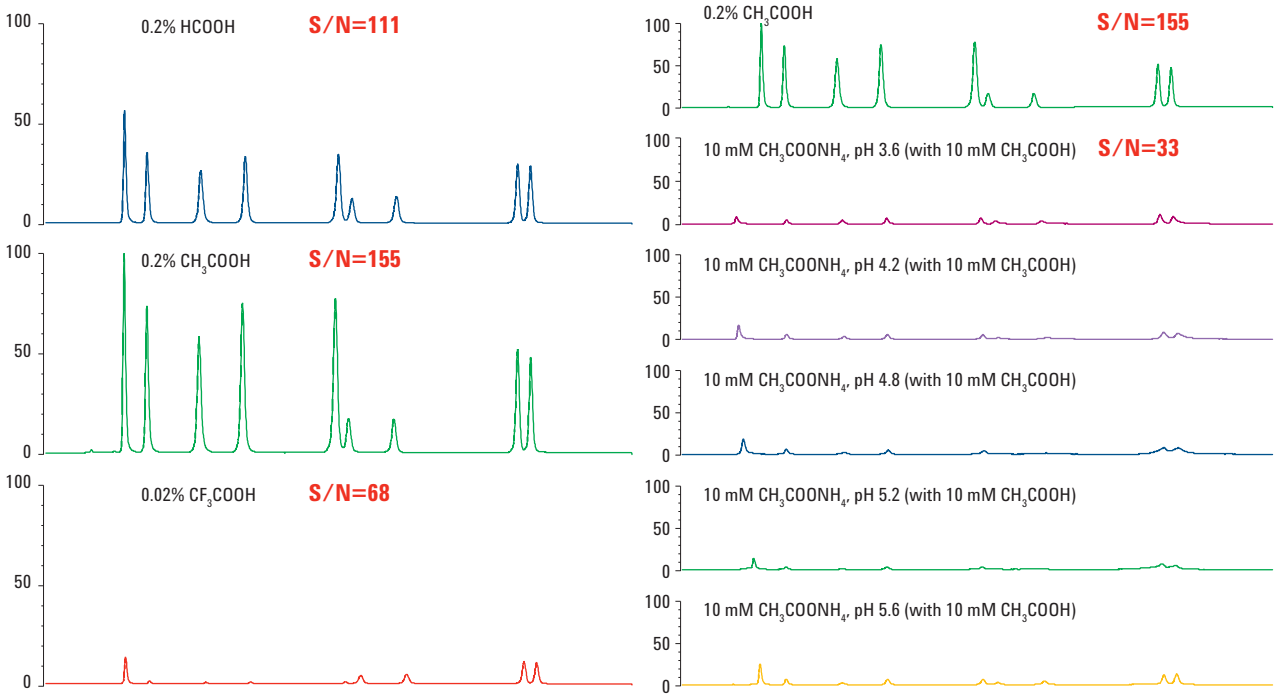


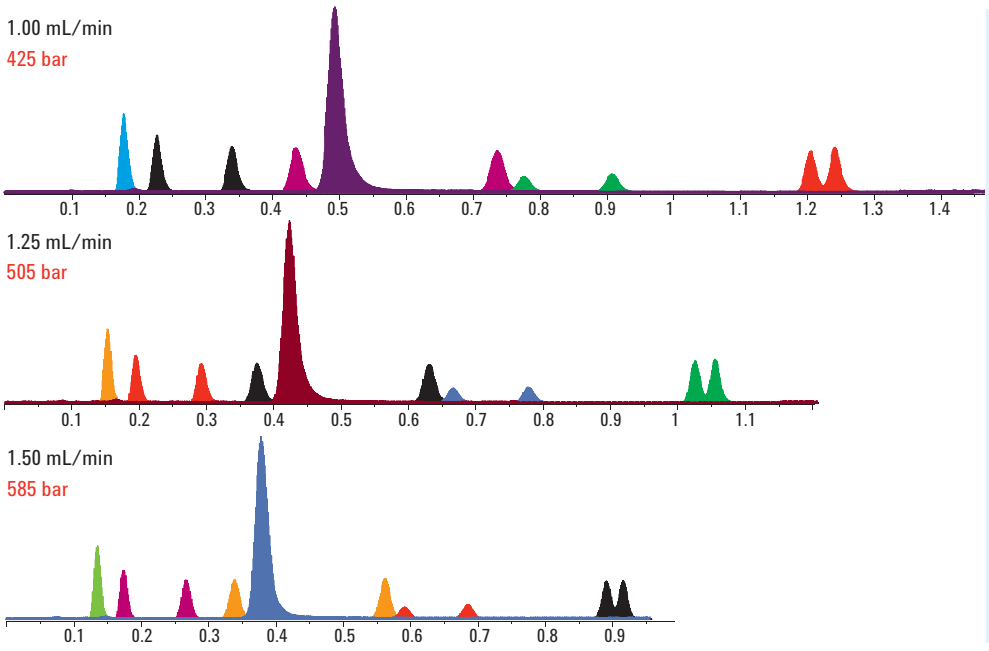
Figure 4. Various MS-friendly mobile phases are screened in order to find a replacement for the H_3PO_4 used in the original LC/UV method (Note: Positive SIM chromatograms of caffeine are not shown, because it is significantly less affected by ion suppression as compared to the catechins.)

EIC overlays in Figure 5 show that because of the lower back pressure generated by the Agilent Poroshell column, more rapid analyses are possible in under 600 bar. A 15 minute method that started out on a 150-mm column, can be reduced to less than 1 minute analysis time on a 50-mm Poroshell 120 column, while preserving the selectivity of the original method. Comparing Figures 3 and 5 shows that the same method (1 mL/min) run on the same column (Agilent Poroshell 120 SB-C18, 2.1 × 50-mm, 2.7-µm) generated notably different back pressures. The difference in back pressure is primarily due to a long piece of small, 0.12 mm, id transfer tubing used to connect the HPLC to the MS. Larger id transfer tubing was not considered for this application as band broadening is likely to occur and would reduce the resolution of this finely tuned, rapid analysis.

Calibration curves for each of the 10 compounds of interest were constructed with a minimum of six points (maximum of 10), while each standard was run in triplicate. Linear regression and correlation coefficient data are shown in Table 2 for all 10 analytes. All curves exhibit a high degree of linearity up to a maximum analyzed amount of 10 ng on column (Poroshell 120 SB-C18, 2.1 × 50 mm). All tea samples were diluted 1:10 with water prior to injection in attempt to not exceed the highest concentration calibration standard. Only one compound, EGCG, in the brewed green tea sample exceeded the maximum concentration after the 1:10 dilution; the concentration of EGCG was extrapolated from the linear regression equation found in Table 2.

Table 2. Calibration data for Catechins and Caffeine; Minimum Six Point Calibration Curve with all Standards Run in Triplicate

	Linear regression line	**Correlation coefficient, R^2**
Gallic acid	y = 0.466 x	0.995
Gallocatechin	y = 0.407 x	0.996
Epigallocatechin	y = 0.355 x	0.996
Catechin	y = 0.601 x	0.996
Caffeine	y = 3.439 x	0.995
Epicatechin	y = 0.638 x	0.995
Epigallocatechin gallate	y = 0.153 x	0.998
Gallocatechin gallate	y = 0.183 x	0.996
Epicatechin gallate	y = 0.396 x	0.998
Catechin gallate	y = 0.371 x	0.996



A	0.2% CH_3COOH in H_2O		
B	CH_3CN		
40 °C			
Column	Agilent Poroshell 120 SB-C18, 2.1 × 50 mm, 2.7 µm		
Source	350 °C, 10 L/min, 50 psi, -/+3500 V		
Acquisition	SIM- (169, 305, 289, 457, 441), SIM+ (195)		
Sample	1.5 µL injection of 0.003 mg/mL each of GA, GC, EGC, C, Caf, EC, EGCG, GCG, ECG, CG in H_2O/CH_3CN		
1.00 mL/min			
Time (min)	0.00	0.50	1.00
%B	10	15	27
1.25 mL/min			
Time (min)	0.00	0.42	0.83
%B	10	15	27
1.50 mL/min			
Time (min)	0.00	0.36	0.71
%B	10	15	27

Figure 5. Catechin analysis is further sped up by increasing flow rate and scaling the gradient according to column volume.

A selection of bottled teas was analyzed, ingredient lists and country of origin for each tea sample are shown in Table 3, and quantitative results are shown in Figure 6. Tea sample A is a Japanese tea that has been stored unopened at room temperature for approximately 3 years. Only gallic acid and caffeine were found in sample A; it is likely that additional catechins were present in this tea originally, but have degraded over time. Bottled tea samples B and D are different brands of Japanese green teas. Both report the same ingredients on their respective labels, and contain approximately the same level of all catechins analyzed in this method. Tea sample C is also a green tea, with the same ingredients reported as B and D, however it is a Taiwanese tea. Compared to the two Japanese green teas, sample C shows a higher concentration of epicatechin gallate and catechin gallate, but a lower concentration of the other eight analytes. Bottled tea samples E and F are Japanese tea blends, both of which contain some amount of green tea according to their labels. Tea E lists barley as its main ingredient, consequently the caffeine and catechin concentrations are all substantially less than the other tea samples. Tea F is an oolong tea blend, which shows a slightly different composition than the green teas. Compared to the two pure Japanese green tea samples B and D, the oolong blend contains more gallic acid and caffeine, similar amounts of epicatechin gallate and catechin gallate, but lower concentrations of the remaining catechin compounds.

Table 3. Ingredient Lists and Country of Origin for Bottled Tea Samples

Bottled tea sample	Country of origin	Ingredients
A	Japan	(unknown)
B	Japan	purified water, green tea, ascorbic acid
C	Taiwan	mineral water, green tea, vitamin C, natural flavor
D	Japan	water, green tea, ascorbic acid
E	Japan	pearl barley, brown rice, sprouted rice, green tea, barley, houttuynia cordata, chickory, quinoa, angelica keiskei, vitamin C
F	Japan	oolong tea, puaru tea, green tea, brown tea, chickory, soybean, sesame, vitamin C

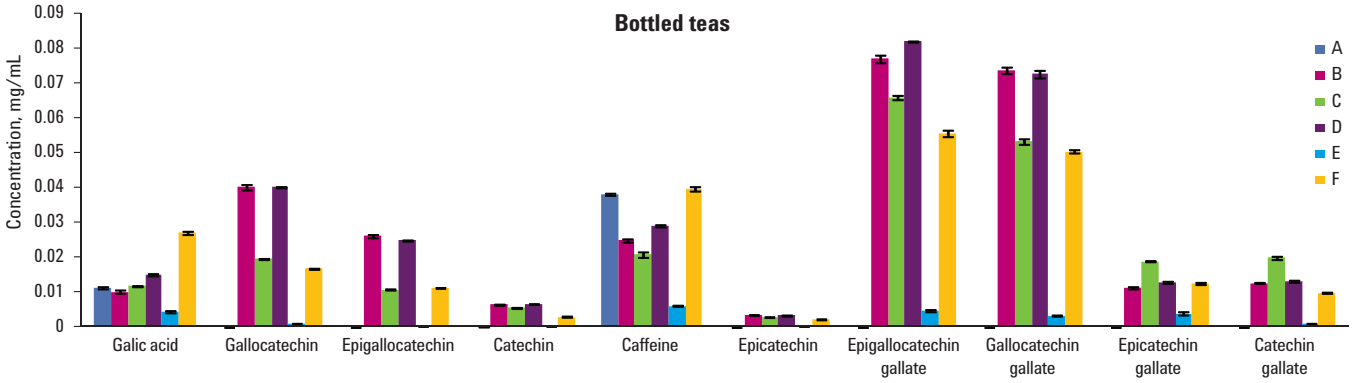


Figure 6. Six bottled tea samples analyzed; 3 green teas (2 Japanese [B,D], 1 Taiwanese [C]), 1 barley tea blend (E), 1 oolong tea blend (F), and 1 unknown (A).

For comparison to the bottled tea samples shown in Figure 6, Figures 7 and 8 show freshly brewed green and black tea samples respectively. Both brewed tea samples show peak concentrations of most compounds when the tea bag is allowed to steep for six to 10 minutes. After this optimal steep time, compounds begin to degrade in both cases. Most notably is epigallocatehin gallate, which degrades by more than 50% of the maximum concentration in 60 minutes. Also interesting regarding epigallocatechin gallate is how much more concentrated it is with the brewed green tea sample than with the bottled green tea samples.

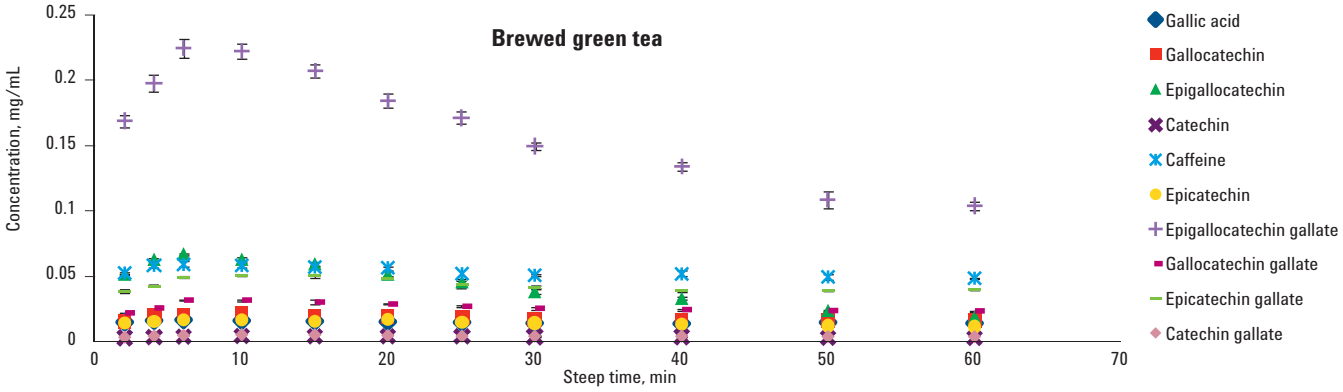


Figure 7. Freshly brewed green tea sample; 1 commercial tea bag steeped in 6 oz initially boiling water, with samples taken over time.

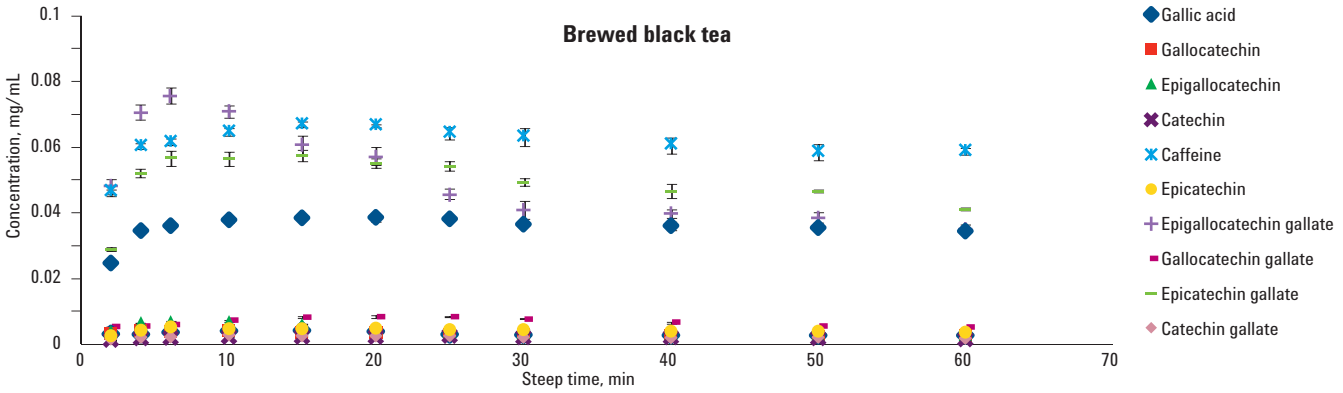


Figure 8. Freshly brewed black tea sample; 1 commercial tea bag steeped in 6 oz initially boiling water, with samples taken over time.

Figure 9 shows a lifetime study of more than 1500 injections of a dirty sample at high pressure (550 bar) without gaining pressure or increasing peak width. The green tea sample was brewed from a commercial tea bag in 6 oz of initially boiling water for six minutes, and then injected directly into the HPLC without any filtration or dilution. The sample was replaced twice daily, as compound degradation was prevalent for the catechins (caffeine was relatively stable). The 2-µm frit found in the Agilent Poroshell 120 SB-C18 (2.1 × 100 mm, 2.7 µm) column is ideal for dirty samples, as it resists plugging more than the 0.5-µm frit found in sub-2 µm columns.

Conclusion

An existing HPLC method for the analysis of catechins in green tea was successfully transferred from totally porous Agilent ZORBAX SB-C18, 1.8-µm to superficially porous Agilent Poroshell 120 SB-C18, 2.7-µm. The selectivity of the two columns is similar enough that no method adjustments were necessary to maintain the 10 compound separation. Highly linear calibration curves were constructed for all compounds, and various bottled and freshly brewed teas were quantified and compared for catechin content. The larger 2-µm frit in the Poroshell 120 column was also exploited in a lifetime test, showing more than 1500 injections of a dirty sample at high pressure without negative effects on chromatography.

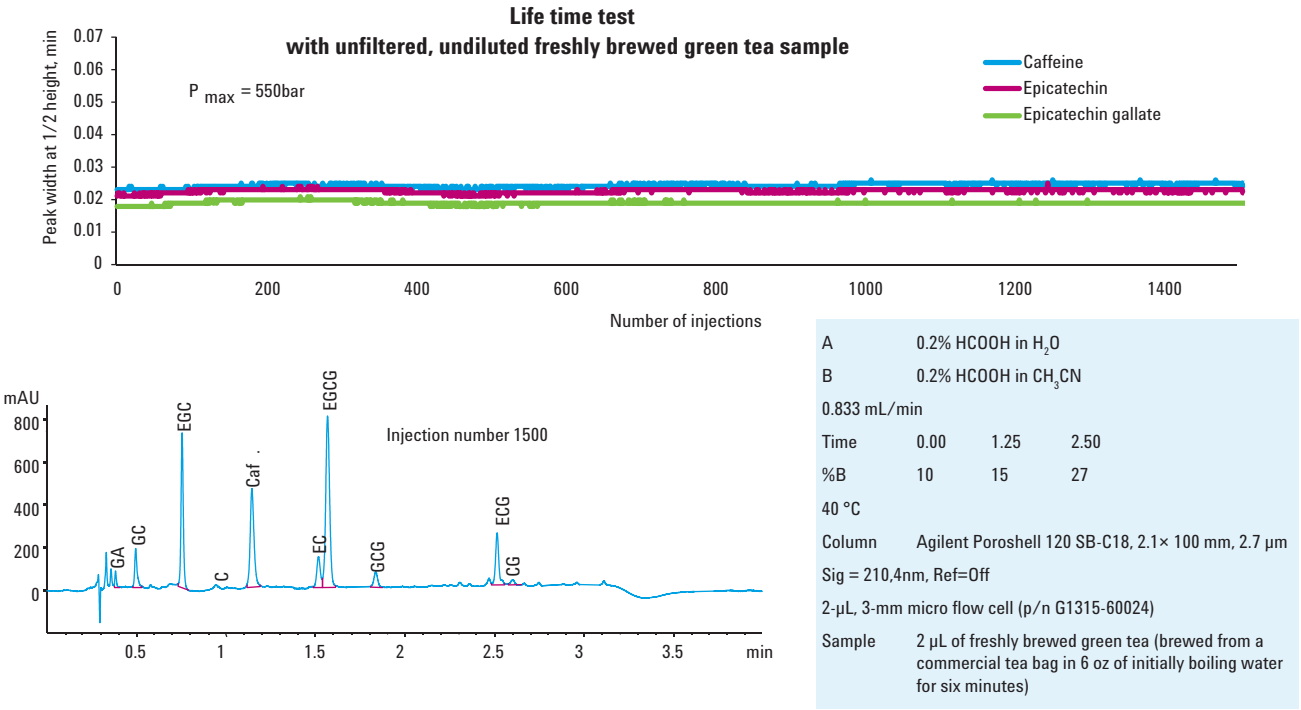


Figure 9. Lifetime study of 1500 injections of an unfiltered, undiluted, freshly brewed green tea sample showing no peak broadening or increase in pressure.

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