

Quantitative Analysis of Catechins in Tea by UHPLC/UV

Guifeng Jiang, Terry Zhang, Thermo Fisher Scientific, San Jose, CA, USA

Goal

Develop and validate robust UHPLC/UV methods for high-throughput screening and quantitation of catechins, and demonstrate applicability of these methods to the analysis of tea matrices.

Introduction

Tea, derived from the leaves of the *Camellia sinensis* plant, is the world's second most popular beverage after water.¹ The health benefits associated with tea are primarily attributed to its catechin constituents. These polyphenols are found in many food products but are present at particularly high concentrations in green tea, which is unfermented. Existing evidence indicates that tea catechins, notably epigallocatechin-3-gallate, are pharmacologically active. Their chemopreventive and cardioprotective effects have been demonstrated in numerous animal studies and some, but not all, human studies.^{1,2} Several catechins exhibit exceptional antimicrobial properties at low nanomolar levels and may prove beneficial in applications such as food protection, the prevention and treatment of infection, and the promotion of oral and digestive health.^{3,4} Furthermore, several studies show that green tea catechins stimulate thermogenesis and fat oxidation, which may have implications for the prevention of obesity.^{5,6} Catechins likely exert their biological effects through multiple and complementary mechanisms of action, including antioxidation, pro-oxidation, and the modulation of cell signaling pathways and transcription factors. Determination of the effects of catechins and tea consumption on human health requires sensitive and robust methods for analysis and quantitation of polyphenols in a range of matrices. Sensitive, rapid and robust analytical methods are also necessary for routine screening and quality assurance of catechin-rich foods.



Tea is a complex matrix and its components are generally analyzed by high performance liquid chromatography (HPLC) coupled with UV detection. The main drawback of conventional HPLC methods for the analysis of tea catechins is the compromise between speed and resolution, resulting in typical analysis times of 20 minutes or longer. Ultra high performance liquid chromatography (UHPLC) enables faster separations and higher resolution through the use of sub-2 μm diameter particles.

The Thermo Scientific Accela UHPLC system offers the flexibility of performing both HPLC and UHPLC separations on a single platform. The Accela™ 1250 quaternary pump delivers accurate and precise flows and gradients over a wide range of flow rates (0.1–2 mL/min) and pressures (1–1250 bar) to accelerate method development and maximize method flexibility. Thermo Scientific 1.9 μm Hypersil GOLD PFP (perfluorophenyl) columns enhance retention and selectivity for UHPLC separations of substituted aromatic compounds. In this application note, we demonstrate fast and robust separation, detection and quantitation of sub-ppm levels of catechins and other phenolic compounds using the Accela LC platform and high performance sub-2 μm columns.



Key Words

- Accela
- Catechins
- Food Analysis
- Phenols
- UHPLC

Materials and Methods

Preparation of Catechin Standard Solutions

Catechin/phenol standards (1 mg/mL) were purchased from Sigma-Aldrich (St. Louis, USA). Stock solution of the standard mixture at 125 mg/L were prepared by mixing the 11 standards, diluting with 60:40 methanol:water (v/v) and used for methods development. Calibration standards at 0.0125, 0.05, 0.125, 0.5, 1.25, 5.0, 12.5, 50 and 125 mg/L levels were prepared by serial dilution of the stock mixture solution.

Preparation of Tea Samples

Two commercially available teas, jasmine green tea and black tea, were examined for catechin content. For each tea, 1 g of tea leaves was placed in 10 mL of 80:20 methanol:water for an hour. The supernatant was diluted 1/10 with 50:50 methanol:water and analyzed directly by LC.

LC Instrumentation

LC separations were performed on an Accela UHPLC system with a 1250 UHPLC pump, an autosampler (Thermo Fisher Scientific, San Jose, CA, USA) and an 80 Hz Accela PDA detector (Thermo Fisher Scientific, San Jose, CA, USA).

LC Parameters

Column	Hypersil GOLD™ C18 PFP columns: 2.1 × 50 mm, 1.9 μm particle size 2.1 × 100 mm, 1.9 μm particle size 2.1 × 150 mm, 1.9 μm particle size 2.1 × 200 mm, 1.9 μm particle size				Gradient III	Column: HG PFP 2.1 × 50 mm, 1.9 μm			
Mobile Phase	A: Water 0.1 % Formic acid B: Methanol 0.1% Formic acid					Time	A %	B %	μL/min
Column Temperature	30 °C					0.00	98.0	2.0	420
Sample Injection Volume	2 μL					0.10	98.0	2.0	420
Gradient I	Column: HG PFP 2.1 × 100 mm, 1.9 μm					3.60	35.0	65.0	420
	Time	A %	B %	μL/min		3.70	5.0	95.0	420
	0.00	98.0	2.0	420		4.50	5.0	95.0	420
	0.10	98.0	2.0	420		4.60	98.0	2.0	420
	7.10	35.0	65.0	420		6.00	98.0	2.0	420
	7.20	5.0	95.0	420	Gradient IV	Column: HG PFP 2.1 × 150 mm, 1.9 μm			
	7.90	5.0	95.0	420		Time	A %	B %	μL/min
	8.00	98.0	2.0	420		0.00	98.0	2.0	420
	10.00	98.0	2.0	420		0.10	98.0	2.0	420
Gradient II	Column: HG PFP 2.1 × 100 mm, 1.9 μm					6.60	35.0	65.0	420
	Time	A %	B %	μL/min		6.70	5.0	95.0	420
	0.00	98.0	2.0	900		7.20	5.0	95.0	420
	0.10	98.0	2.0	900		7.30	98.0	2.0	420
	2.80	35.0	65.0	900		9.00	98.0	2.0	420
	3.10	5.0	95.0	900	Gradient V	Column: HG PFP 2.1 × 200 mm, 1.9 μm			
	3.50	5.0	95.0	900		Time	A %	B %	μL/min
	3.51	98.0	2.0	900		0.00	98.0	2.0	420
	5.00	98.0	2.0	900		0.10	98.0	2.0	420
						11.10	35.0	65.0	420
						11.20	5.0	95.0	420
						13.00	5.0	95.0	420
						13.10	98.0	2.0	420
						16.00	98.0	2.0	420

Results and Discussion

UHPLC Separation of Catechin Standards

The use of sub-2 μm particle columns facilitates rapid analysis of complex samples by improving chromatographic resolution, speed and sensitivity. Columns with perfluorophenyl functionalities help to enhance retention and improve resolution when separating difficult or complex mixtures of substituted aromatic compounds. Using the Accela UHPLC system, a Hypersil GOLD PFP column (1.9 μm, 2.1 × 100 mm) and a simple water/methanol gradient (Gradient I), a standard mixture containing five catechins and six other phenolic compounds was separated and detected under 7 minutes (Figure 1). With the exception of catechin gallate and anisic acid, all analytes were baseline-resolved with an elution order of gallic acid, 3,5-dihydroxybenzoic acid, epigallocatechin, caffeine, epicatechin, epigallocatechin gallate, epicatechin gallate, anisic acid, catechin gallate, 3,4,5-trimethoxycinnamic acid, and myricetin. As the majority of aqueous and matrix contaminants usually elute early at void volume, elution of the first catechin, epigallocatechin at 3.86 minute ensures a robust quantitation method. For this analysis, a flow rate of 420 μL/min was used, generating a backpressure of 600 bar.



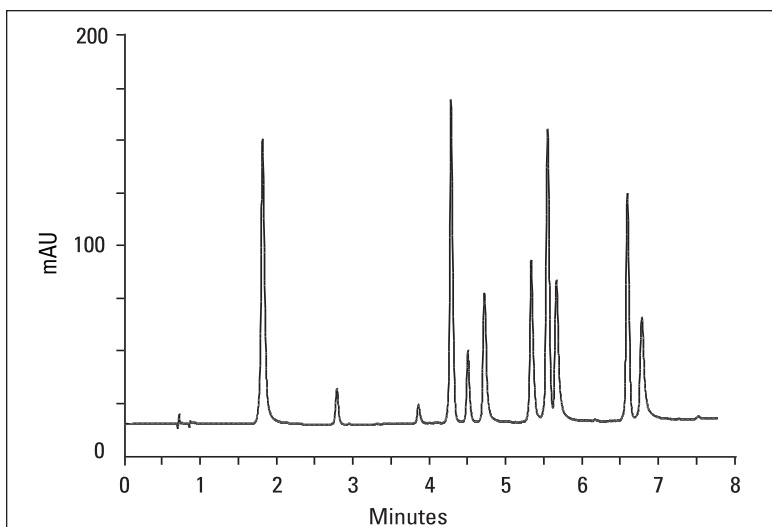


Figure 1: UHPLC separation of a mixture of phenolic compounds containing catechin standards (125 ppm). Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 $\mu\text{L}/\text{min}$. UV detection: 275 nm.

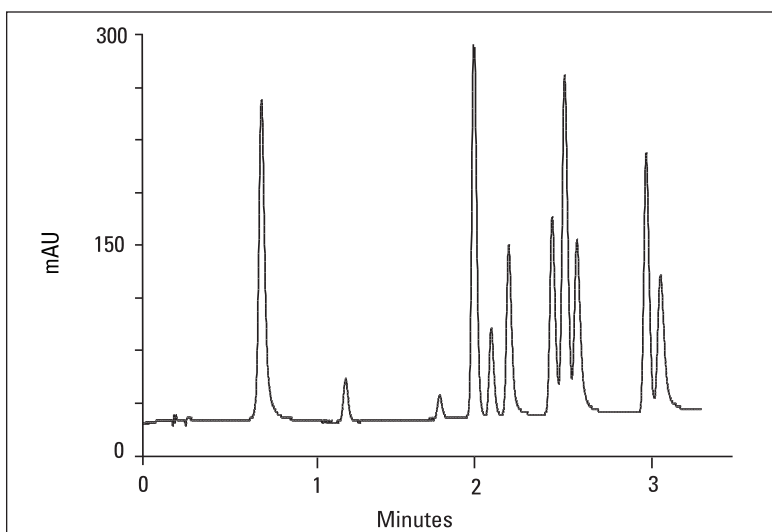


Figure 2: Effect of increasing flow rate. Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 900 $\mu\text{L}/\text{min}$. Pressure: 1200 bar. UV detection: 275 nm.

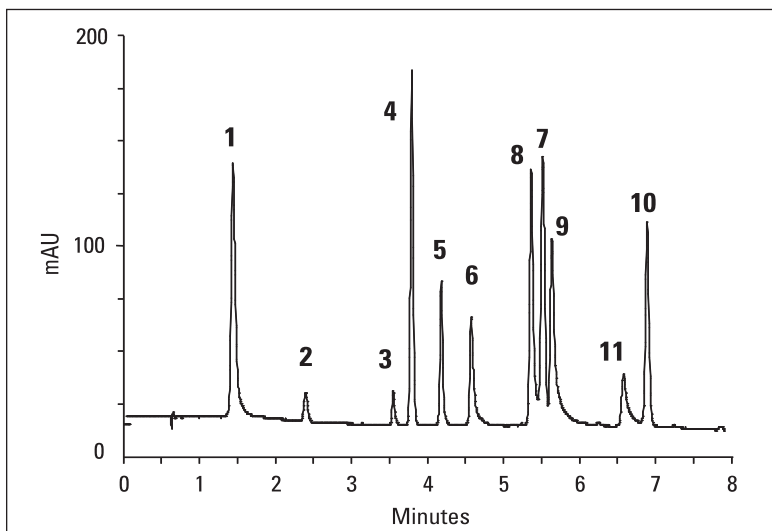


Figure 3: UHPLC separation of a mixture containing catechin standards (125 ppm) using a water/acetonitrile gradient. Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1 mm. Flow rate: 420 $\mu\text{L}/\text{min}$. UV detection: 275 nm.

Effect of Flow Rate

Compared to larger (5 and 3.5 μm) particles, sub-2 μm particles are less affected by flow rate, therefore faster flow rates, within the pressure limit of the LC system, may be used to increase sample throughput without detrimental effects on peak efficiency. Figure 2 demonstrates that increasing the flow rate to 900 $\mu\text{L}/\text{min}$ decreases the analytical run time from approximately 7 minutes to under 3 minutes (Gradient II). At this flow rate, backpressures as high as 1200 bar were generated. While this 3-minute separation is ideal for high-throughput analysis, some degradation in resolution is observed; hence the lower flow rate (420 $\mu\text{L}/\text{min}$) separation was selected as the method for robust quantitation.

Effect of Mobile Phase

The mobile phase composition has a significant effect on analyte retention and the quality of separations. Specifically, the influence of methanol and acetonitrile mobile phases on the UHPLC separation was investigated. Baseline resolution and excellent peak shapes were achieved with a water/methanol mobile phase (Figure 1). In contrast, a water/acetonitrile mobile phase resulted in asymmetrical peak shapes for some compounds (Figure 3) Elution orders of the compound 7, 8 and 10 and 11 (listed in table 1) are reversed. While water/methanol is clearly the superior mobile phase for this separation, its 1.5- to 2-fold higher viscosity compared to water/acetonitrile mixtures results in significantly higher backpressures, which is an important consideration in LC method development.

Effect of Column Length

Increasing the length of the column increases the number of plates and enhances resolution (Figure 4), but also increases analysis times and column backpressures (Figure 5). Unlike some LC systems, the Accela platform provides the same high level of performance at 300 bar as at 1200 bar. As shown in Figure 5, sub-2 μm columns of 20 cm in length

can be used with highly viscous water/methanol mobile phases without exceeding the upper pressure limits of the Accela 1250 pump. The 10 cm-long column was chosen for this application because it provides both the necessary separation speed and resolution required for high-throughput screening and quantitative analysis.

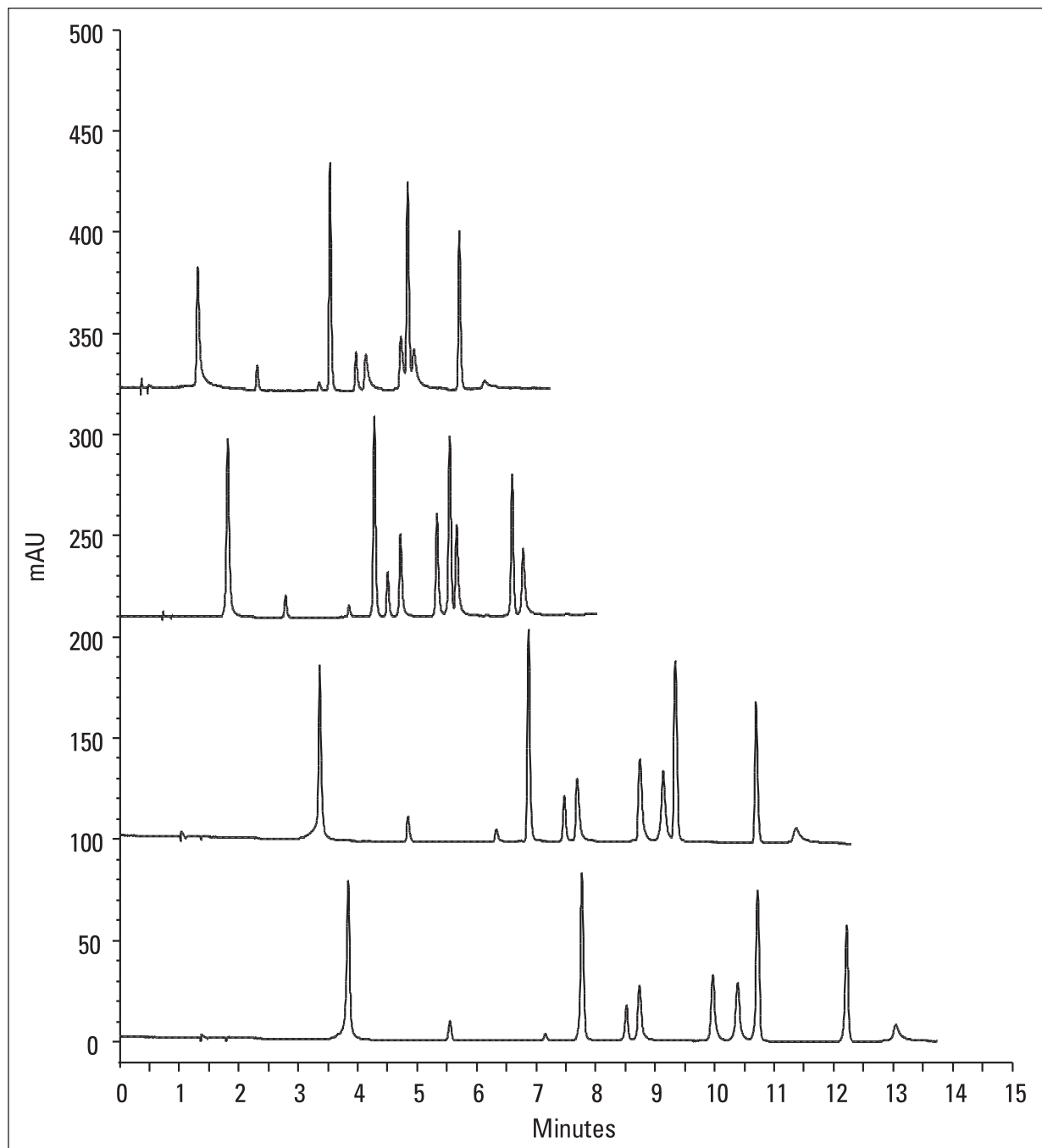


Figure 4: The effect of column length on the UHPLC separation of a mixture containing catechin standards. Mobile phase: Column: 1.9 μm Hypersil GOLD PFP. Flow rate: 420 $\mu\text{L}/\text{min}$. UV detection: 275 nm.

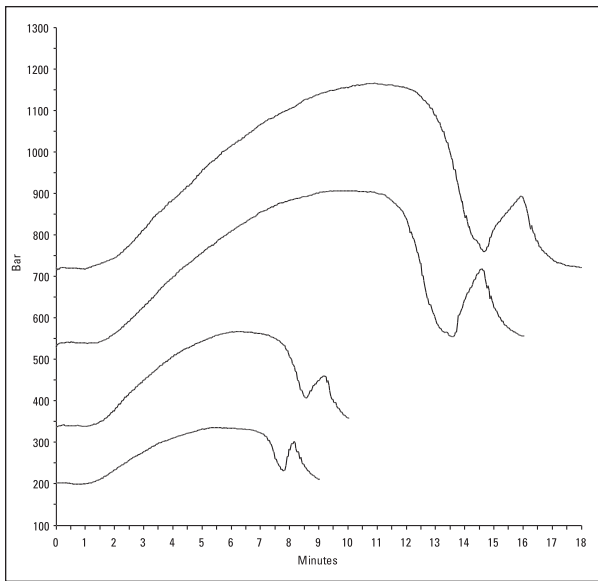


Figure 5: The generated backpressure as a function of column length. Flow rate: 420 $\mu\text{L}/\text{min}$.

Validation of UHPLC High-throughput and Quantitation Methods

Reproducibility of the high-throughput method (900 $\mu\text{L}/\text{min}$, 3 minute analytical run) was investigated by analyzing six replicate injections of each analyte (Figure 6). Retention time RSDs ranged from 0.04–0.18% while peak area RSDs ranged from 0.20–0.91% (Table 1), indicating excellent method reproducibility at 1200 bar, particularly of the UHPLC pump.

Peak Name	RSD% RT	RSD% Area
1. Gallic Acid	0.18	0.66
2. 3,5-dihydroxybenzoic acid	0.14	0.57
3. Epigallocatechin	0.05	0.91
4. Caffeine	0.06	0.20
5. Epicatechin	0.05	0.31
6. Epigallocatechin Gallate	0.07	0.20
7. Epicatechin Gallate	0.07	0.47
8. Anisic Acid	0.07	0.31
9. Catechin Gallate	0.08	0.49
10. 3,4,5-Trimethoxycinnamic Acid	0.04	0.77
11. Myricetin	0.06	0.99

Table 1: Reproducibility of the High-throughput UHPLC Method

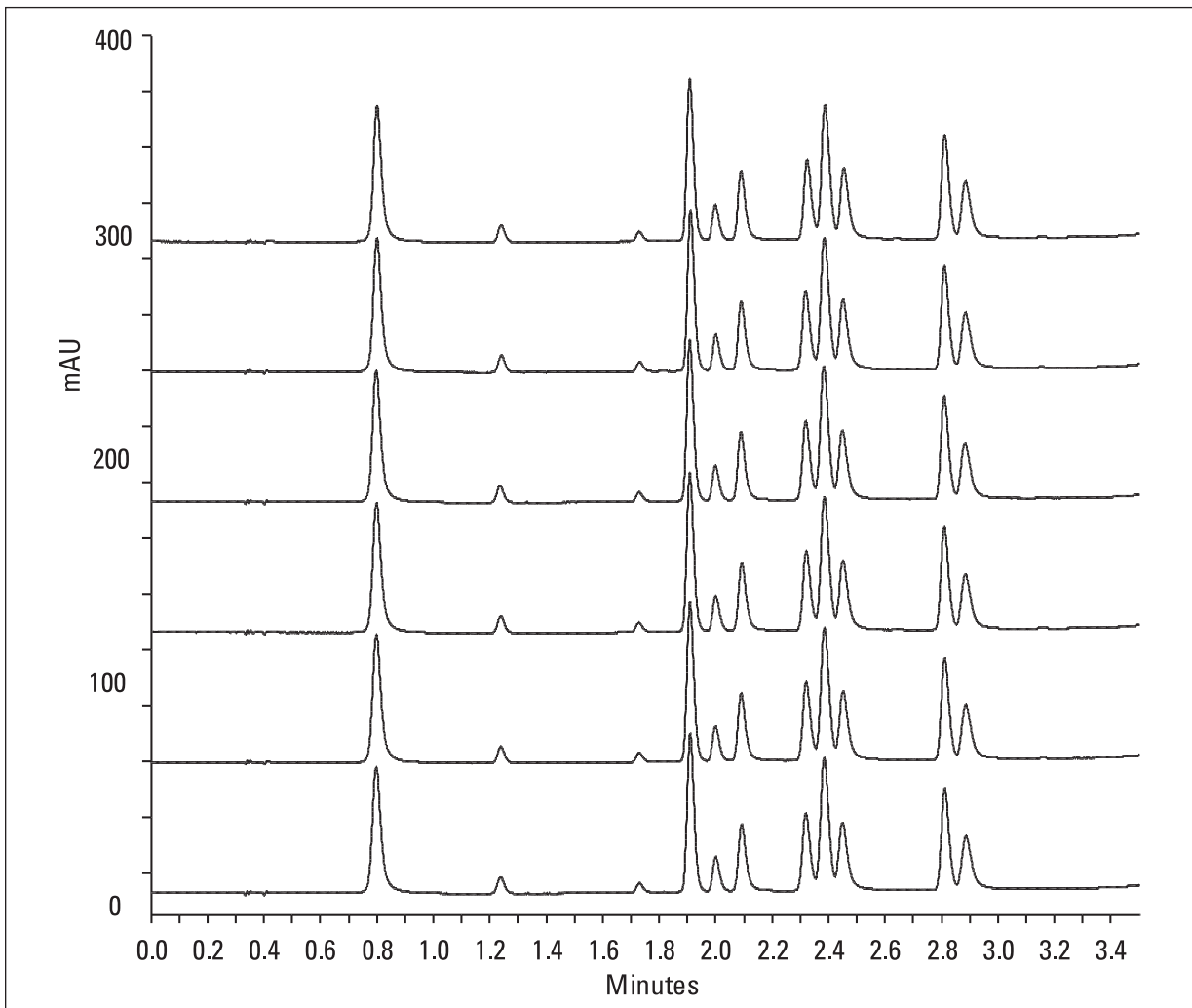


Figure 6: Reproducibility of the high-throughput UHPLC method (900 $\mu\text{L}/\text{min}$) was assessed by analyzing six replicate injections of each analyte. Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 900 $\mu\text{L}/\text{min}$. Pressure: 1200 bar. UV detection: 275 nm.

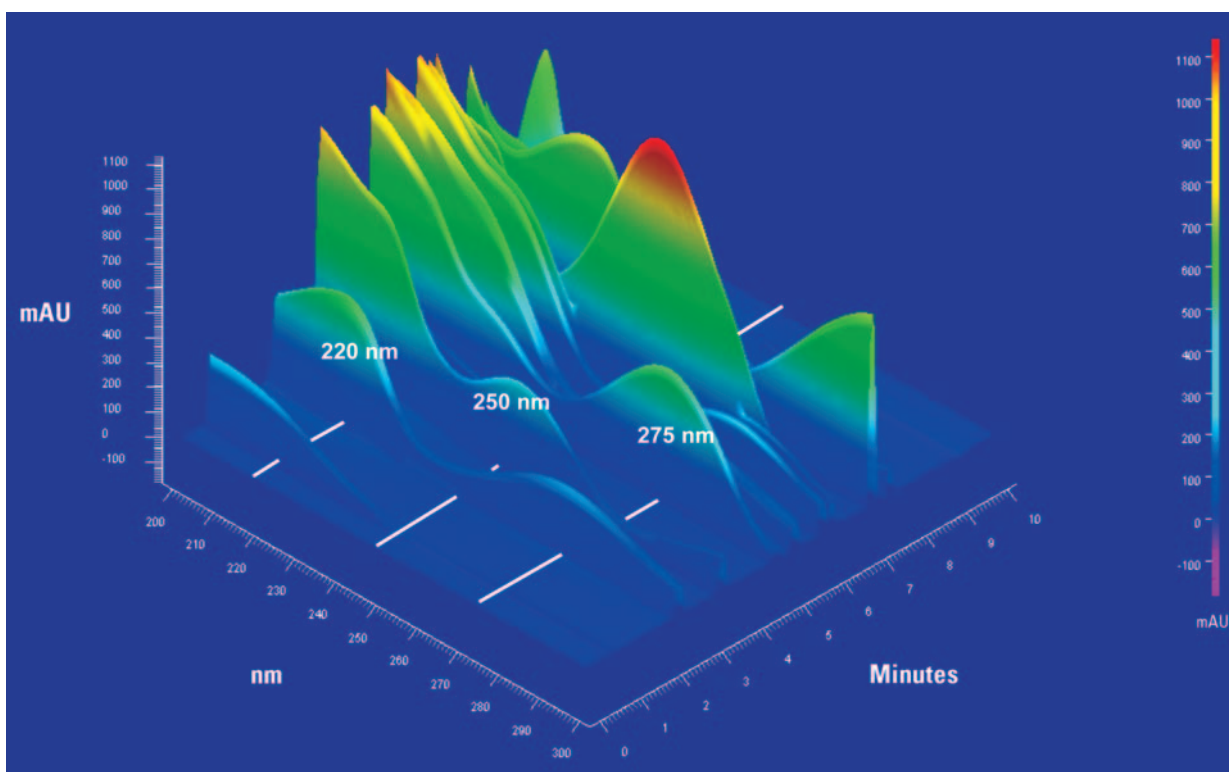


Figure 7: Selection of the wavelength for quantitation was based on the best S/N for all analytes rather than on the most intense wavelength (λ_{max}) for most analytes.

Quantitative Analysis

The optimal wavelength for quantitation, 275 nm, was determined based on the best signal-to-noise ratio (S/N) for all analytes, and not on the most intense wavelength, in order to ensure the lowest limits of detection (LODs) and limits of quantitation (LOQs) (Figure 7). The UHPLC quantitation method (Gradient I) exhibited excellent reproducibility, with peak area RSDs in the range

0.20-0.91%. (Table 2). Excellent linearity in detector response was observed over three orders for catechin and the other phenolic standards, with correlation coefficients greater than 0.99 for all analytes (Table 2). LODs and LOQs, defined as S/N ratio of 3 and 10, respectively, are shown in Table 2. LODs ranged from 0.11 to 9 mg/L, and LOQs ranged from 0.34 to 28 mg/L.

Compound	RSD% Area at 50 ppm	Linearity Range mg/L	Correlation Coefficients	LOQ mg/L	LOQ mg/L
Gallic Acid	0.39	0.5–125	0.9993	0.1	0.4
3,5-Dihydroxybenzoic Acid	0.51	1.25–125	0.9999	0.5	1.6
Epigallocatechin	0.79	12.5–1000	0.9999	9.0	28.0
Caffeine	0.17	0.125–125	0.9999	0.1	0.2
Epicatechin	0.13	0.5–125	0.9983	0.5	1.6
Epigallocatechin Gallate	0.29	0.5–125	0.9954	0.2	0.6
Epicatechin Gallate	0.73	0.5–125	0.9984	0.2	0.5
Catechin Gallate	0.97	0.5–125	0.9993	0.2	0.6
Anisic Acid	0.30	0.125–125	0.9999	0.1	0.2
3,4,5-Trimethoxycinnamic Acid	0.06	0.125–125	0.9999	0.1	0.3
Myricetin	0.54	5–125	0.9993	0.5	1.7

Table 2: Validation parameters of the UHPLC quantitation method. Column: 1.9 μ m Hypersil GOLD PFP, 100 \times 2.1mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 μ L/min. Pressure: 600 bar. UV detection: 275 nm.

Compound	Jasmine (mg/g)	RSD% (n=3)	Black Tea (mg/g)	RSD% (n=3)
Gallic Acid	0.7	0.6	0.8	0.8
3,5-Dihydroxybenzoic Acid	N.D.	-	N.D.	-
Epigallocatechin	51.3	0.5	17.5	1.9
Caffeine	15.7	0.3	10.6	0.7
Epicatechin	4.1	0.4	1.0	1.8
Epigallocatechin Gallate	13.7	0.4	2.0	0.1
Epicatechin Gallate	15.1	0.4	2.7	0.5
Catechin Gallate	0.3	0.4	N.D.	-
Anisic Acid	0.06	0.1	N.D.	-
3,4,5-Trimethoxycinnamic Acid	0.3	0.2	0.7	0.2
Myricetin	0.9	0.4	N.D.	-

Table 3: Quantitation of catechins and other phenols in tea extracts. Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 $\mu\text{L}/\text{min}$. Pressure: 600 bar. UV detection: 275 nm.

Determination of Catechin Content in Tea Matrices

The UHPLC methods developed and validated for high-throughput screening and quantitation of phenolic compounds were used to analyze and quantitate catechin content in two commercially available teas. Figure 8 shows the UHPLC separations of catechins and other phenols in standard solution and in jasmine and black tea matrices using the high-throughput (900 $\mu\text{L}/\text{min}$) method. The overlaid pressure traces of these three samples demonstrate the exceptional stability of the Accela pump at 1200 bar.

Table 3 summarizes the concentrations of catechins and other phenols detected in the jasmine and black tea samples using the quantitation (420 $\mu\text{L}/\text{min}$) method. Most analytes were detected at sub-ppm levels, and reproducibility, as determined from three replicate injections, was excellent, with RSDs generally below 1%. All five catechins (catechin gallate, epigallocatechin, epicatechin, epigallocatechin gallate, and epicatechin gallate) were present in jasmine green tea, whereas only four were detected in black tea. In general, jasmine green tea contained significantly higher concentrations of catechins and other phenolic compounds compared to black tea. These differences are likely attributed to differences in the way green and black teas are processed. Unlike green tea, black tea is fermented, a process that causes oxidative degradation of catechins and other phenols.

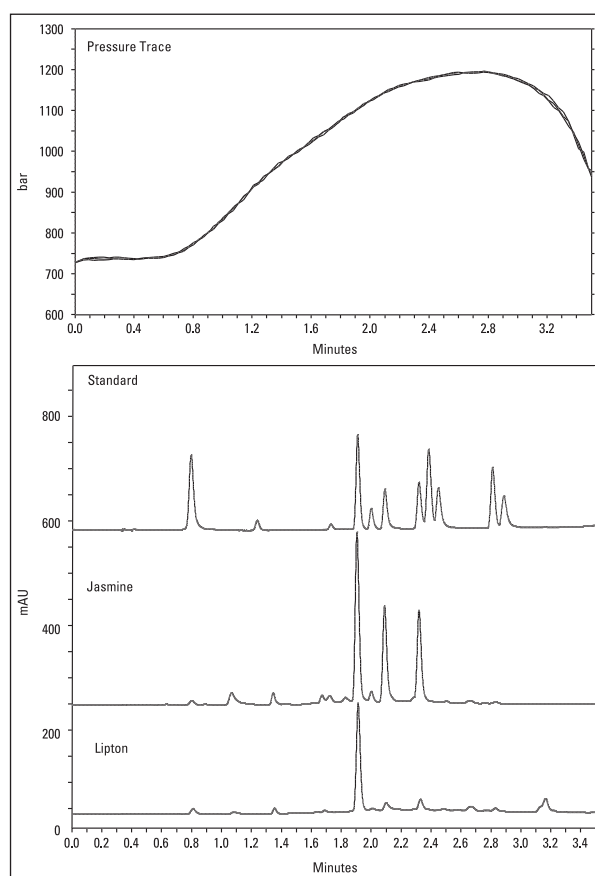


Figure 8: UHPLC separation and detection of catechins and other phenolic compounds in a standard solution and in jasmine and Lipton tea matrices. Column: 1.9 μm Hypersil GOLD PFP, 100 \times 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 900 $\mu\text{L}/\text{min}$. Pressure: 1200 bar. UV detection: 275 nm.

Conclusion

Robust UHPLC methods for high-throughput screening and quantitation of sub-ppm levels of catechins and other phenolic compounds were developed using the Accela UHPLC system. Several method parameters – mobile phase composition, flow rate and column length – were investigated in order to optimize separation speed and resolution for high-throughput and quantitation applications. Using these UHPLC methods, significant differences in catechin and phenolic content in green and black tea samples were found. Although these methods were applied for the analysis of catechins and phenols in tea, they can be adapted for analysis of other foodstuffs as well as for use in studies examining the effects of catechin intake on human health.

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