

Article

The Use of Liquid Chromatography and Mass Spectrometry to Identify and Quantify Chemical Components in Tea Extracts

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Abstract: A fast, straightforward, efficient, and high throughput reverse-phase high-performance liquid chromatography and electrospray ionization mass spectrometry method has been developed to analyze tea extracts containing polyphenols. The polyphenols detected in green tea (GT), black tea (BT), and paradise tropical tea (PTT) were separated in 18 min at wavelengths 254 nm, 260 nm, and 280 nm with a gradient elution on the RP-HPLC system. Our approach detected, identified, and quantified three catechins in the tea extracts analyzed, and the response at 280 nm wavelength was the best. The ESI-MS data confirmed the fragmentation patterns of the catechins detected in the tea extract. The validation data showed that the limit of detection (LOD) and limit of quantitation (LOQ) of catechins ranged from 5.26 ± 0.02 to 36.44 ± 0.02 ppb, and 17.52 ± 0.03 to 121.45 ± 0.16 ppb, respectively, for six catechins studied. The standard addition calibration approach used to quantify the catechin content in the tea extract simultaneously showed that PTT has a higher catechin content than GT and BT. The content of polyphenols in GT, BT, and PTT are summarized. This approach holds great promise for quality control studies to quantify polyphenols in nutritional products.

Keywords: Reverse-phase high-performance liquid chromatography, Electrospray ionization mass spectrometry, Standard addition calibration, Polyphenols, Catechins, Tea extracts

Introduction

Reports have continuously documented that green tea (GT) and black tea (BT) are the most widely consumed beverages worldwide ¹⁻³. Other tea samples, such as paradise tropical tea (PTT), are becoming popular. The PTT comprises 100% black tea and natural and artificial flavors. It has been reported that tea samples have potential health benefits, including inhibiting cancer and preventing cardiovascular disease ⁴⁻⁷. Most tea samples contain polyphenols giving them their antioxidant and the claimed therapeutic properties ^{8,9}. However, polyphenols vary according to tea variety, climate, and cultivation method ¹⁰⁻¹². Given the varied report of polyphenols or

catechin amounts in different tea samples, it is essential to develop a robust analytical method to assess, quantify, and differentiate catechins in tea sample extracts ¹³.

In reverse-phase high-performance liquid chromatography (RP-HPLC), C_{18} columns are usually coupled to UV, DAD, UV-DAD, fluorescence, or electrochemical detectors and have been the most widespread method for the determination of polyphenols in plant samples ^{14,15}. However, published studies have reported an analysis time of up to 60 minutes per sample ^{16,17}. In cases where detailed structural information is required, especially for studying unknowns, techniques with high sensitivity and

selectivity may be needed. Some investigations have utilized RP-HPLC-UV-DAD and AP-ESI-MS for this purpose¹⁸. Recently, work has focused on separating tea catechins by HPLC, resulting in an excellent performance with low detection and quantitation limits¹⁹. An improved method that investigated three RP-HPLC columns with an analysis time of 30 minutes for one of the columns was developed to separate Miang catechins²⁰. Other recent approaches have developed more straightforward extraction methods that are closely compared to the ISO 14502-2 method for extracting and analyzing matcha tea using HPLC²¹.

On the other hand, mass spectrometry (MS) has long been used to decipher organic structures²². MS has served for decades as the most powerful detector for chromatography, offering qualitative and quantitative information, providing high sensitivity, and distinguishing different substances with the same retention time. LC-MS can serve as a critical tool for guarding the safety of our food supply by monitoring toxic substances such as pesticide residues^{23,24}. Several other studies have investigated the HPLC-MS approach for analyzing polyphenols in tea extracts^{25,26}.

Our study aims to develop and validate an RP-HPLC method for simultaneously determining catechins in three tea samples. We also aim to utilize ESI-MS to confirm the fragmentation patterns of catechins detected in the tea extracts. Our approach will be helpful for quality control authorities seeking to quantify active compounds in nutrition products.

Experimental section

Tea sample pretreatment

Dried black tea (Walmart, Winston Salem, NC), Bigelow green tea (Walmart, Winston Salem, NC), and paradise tropical tea (Target Grocery, Winston Salem, NC) leaves were ground in a food processor for approximately one minute, and ~2 g was transferred into a clean 250 mL KIMAX Kimble glass bottle. Each sample was soaked in 5 mL of methanol for three days. After the mixture was filtered with filter paper, all solvent was dried with a roto evaporator. The dried extract

was weighed and dissolved in 1 mL of methanol. The hexane extract was obtained with Soxhlet extraction, and the resulting extract was dried with a roto evaporator. The dried extract was weighed and dissolved in 1 mL of hexane. The resulting solutions were then used to prepare 10 ppm of the sample. Solid-phase extraction (SPE) was used to clean the sample before analyzing it on an Agilent 1260 high-performance liquid chromatography (HPLC) and Advion expression^L CMS mass spectrometry (MS). All materials and reagents used in this investigation are listed in the supplementary instructions section. Fig. 1 shows possible structures of polyphenols that could be found in tea extracts.

Catechin stock solutions & method validation studies

Stock solutions of catechins were prepared to 1000 ppm by accurately weighing 1 mg of analyte and dissolving in 1 mL ethanol. Subsequent serial dilutions from the stock using 9:1 methanol:water were prepared between concentrations of 0.05 ppm to 100 ppm for the six catechins. Calibration studies were conducted by injecting three replicates of each concentration on the Agilent 1260 HPLC to determine method validation parameters. The method validation studies were conducted at 280 nm wavelength. A standard addition calibration curve corresponding to stock solutions of 0.1 to 100 ppm was generated to determine the content of catechins in the tea samples. The stock solution of catechins used in the standard addition calibration curve was a mixture of GC, CH, and ECG. Table S1 shows the experimental design of the standard addition calibration studies.

Agilent 1260 LC & Advion CMS MS instrumental conditions

We analyze the tea extracts dissolved in either methanol or hexane (depending on the solvent used for the initial extraction) on an Agilent 1260 HPLC-DAD instrument. Table S2 summarizes all the experimental operating parameters developed on the Agilent 1260 HPLC-DAD and Advion expression^L CMS MS instrumentations.

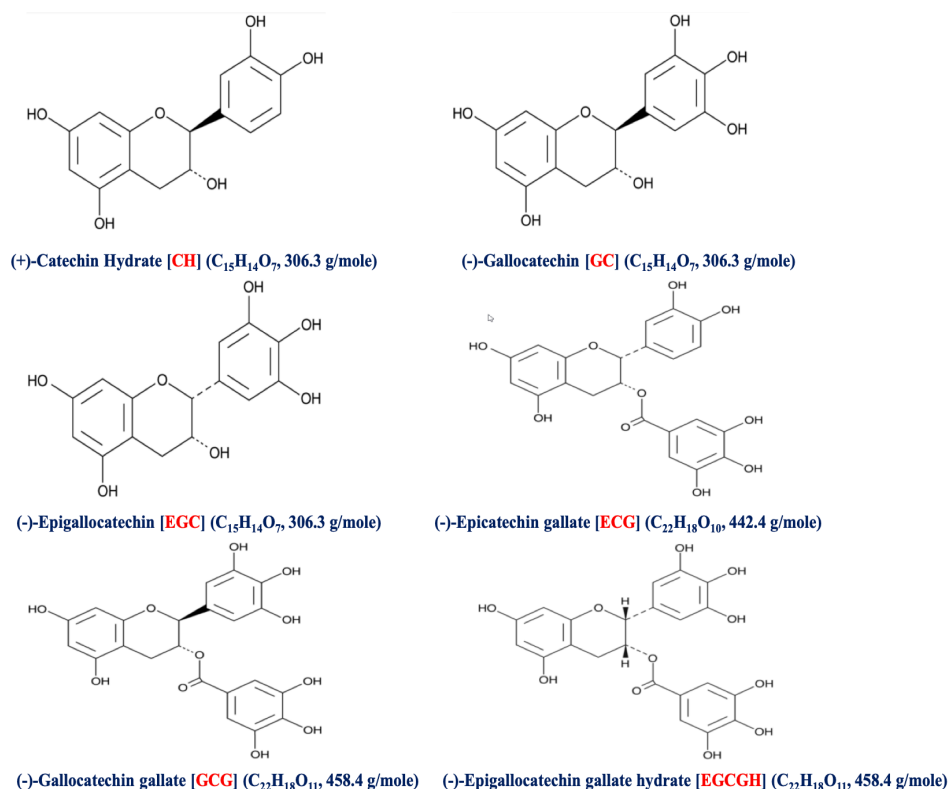


Figure 1. The chemical structures of polyphenols investigated in this study

Results and discussions

Agilent 1260 HPLC-DAD

In high-performance liquid chromatography (HPLC), high pressure is forced through a closed column containing fine particles, and the result is high-resolution separations²⁷⁻²⁹. The Agilent 1260 HPLC used in these studies consisted of an autosampler, a solvent delivery system, a high-pressure chromatography column, and a DAD Detector³⁰. The tea samples were separated with a total run time of 18 min (including 1 min equilibration time) and most polyphenol peaks detected were well resolved within 8 min³¹. Fig. S1 shows example chromatograms of a PTT sample. Three wavelengths, 254 nm, 260 nm, and 280 nm, were used to detect the tea samples. BT extracted with methanol at 254 nm shows five peaks distinct from the blank at retention times, 0.83 ± 0.03 , 4.48 ± 0.03 , 6.53 ± 0.02 , 6.69 ± 0.01 , 6.93 ± 0.02 minutes (see Table S3). Most tea samples contain polyphenols, and the major tea polyphenols are catechins. BT methanol extract studied at 254 nm contains CH (peak at $4.48 \pm$

0.03 min). The peak at 6.53 ± 0.02 min matched the standard peak of ECG. The other three peaks at this wavelength were unidentified. At 260 nm, a total of five peaks were detected. Three peaks matched GC (2.91 ± 0.01 min), CH (4.44 ± 0.02 min), and ECG (6.51 ± 0.02 min). At 280 nm, a total of seven peaks were detected (Table S3). We note that the intensity of peaks increased at 280 nm, indicating 280 nm was the best wavelength of detection. Two peaks from the seven detected matched GC (2.92 ± 0.01 min) and CH (4.42 ± 0.03 min). BT extracted with hexane at 254 nm shows three peaks at retention times 0.91 ± 0.04 , 4.61 ± 0.02 , 12.78 ± 0.03 min (see Table S3). One peak of the three detected matched CH (4.61 ± 0.02 min). The other two wavelengths, 260 nm, identify CH at 4.66 ± 0.03 min and 280 nm at 4.73 ± 0.02 min. We note that the CH peak shows a weaker response in the hexane extract than that detected in the methanol extract. In either case, the 280 nm wavelength shows a much stronger response.

GT extracted with methanol detected seven

peaks distinct from the blank at 0.84 ± 0.03 , 2.82 ± 0.04 , 4.38 ± 0.03 , 6.44 ± 0.03 , 6.51 ± 0.02 , 6.71 ± 0.02 , 6.86 ± 0.01 min (see Table S3). Three of the peaks match GC (2.82 ± 0.04 min), CH (4.38 ± 0.03 min), and ECG (6.51 ± 0.02 min). We detected the same three peaks at 260 nm, but only GC and CH were detected at 280 nm. With the GT hexane extract, only the ECG response was detected. The response was weak at 254 nm and became stronger at 280 nm.

PTT extracted with methanol detected eight peaks distinct from the blank at 2.89 ± 0.02 , 4.38 ± 0.03 , 5.46 ± 0.02 , 6.51 ± 0.03 , 6.69 ± 0.02 , 6.85 ± 0.03 , 7.24 ± 0.01 , 7.31 ± 0.04 min. As with the other tea samples, the same three polyphenol components (GC [2.89 ± 0.02 min], CH [4.38 ± 0.03 min], and ECG [6.51 ± 0.03 min]) detected in BT and GT were seen in PTT. The ECG response in PTT was stronger than in the other two tea samples. However, two unidentified peaks appear at 6.69 ± 0.02 min and 6.85 ± 0.03 min, with much higher intensity than the other two tea samples was observed in the paradise tropical tea sample. Both GC and CH were detected with the hexane extract at all wavelengths. This study shows that CH was the most abundant catechin detected in all three tea sample extracts. As with previous studies ¹, the HPLC method designed to separate tea catechins saw the non-epi forms (GC and CH) eluted before the epi-forms (ECG). Furthermore, RP-HPLC with acetonitrile as the solvent demonstrates selectivity reported in the past on conventional C_{18} columns ³²⁻³⁷.

Advision expression CMS MS

The MS was attentively tuned daily in positive and negative ion detection modes during these studies. Masses identified for BT extracted with methanol are shown in Table S3. Several m/z were detected for BT extracted with methanol, as shown in Table S3. The most critical masses that correspond to fragmentation from CH in the positive ion mode were 291.3, corresponding to the $[M + H]^+$ ion ($C_{15}H_{15}O_6^+$, 291.3), 108.1 ($C_6H_4O_2^+$), 138.1 ($C_7H_6O_3^+$), 152.5 ($C_8H_8O_3^+$), and 182.8 ($C_9H_{10}O_4^+$). Another catechin detected in the methanol extract was ECG. The most critical masses for ECG were the $[M + H]^+$ ion

($C_{22}H_{19}O_{10}^+$, 443.5), and fragments at m/z 125.2 ($C_6H_5O_3^+$), 291.1 ($C_{15}H_{15}O_6^+$), 303.3 ($C_{15}H_{11}O_7^+$), 317.2 ($C_{16}H_{13}O_7^+$), and 333.2 ($C_{16}H_{13}O_5^+$). The fragments observed at m/z 273.3 and 299.3 were attributed to fragments at 291.1 ($C_{15}H_{15}O_6^+ - H_2O$) and 317.2 ($C_{16}H_{13}O_7^+ - H_2O$) losing H_2O , respectively ¹. The molecular ion in the positive ion mode occurred at m/z 158.6, an unnamed peak. For ECG, a fragment at m/z 125.3 ($C_6H_5O_3^-$) and 305.5 ($C_{12}H_{13}O_7^-$) were observed in the negative ion mode ¹. The peak at m/z 125 was much more intense in the negative ion mode with a ratio of about negative:positive = 10:1. The molecular ion was at m/z 151.3. The only peaks that matched a CH fragmentation in the negative ion mode were 137.1 ($C_7H_5O_3^-$) and 151.3 ($C_8H_7O_3^-$). We also assigned a peak at m/z 305.5 to $[M - H]^-$ ion corresponding to GC. The only other fragment ion of GC in the methanol extract of black tea was the m/z at 106.9, corresponding to a fragment of $C_6H_3O_2^+$. This peak is attributed to a fragment of 107.1 in GC. Several critical fragments corresponding to ECG were detected in the hexane extract of black tea. The most critical was the $[M + H]^+$ ion ($C_{22}H_{19}O_{10}^+$, 443.4), fragments at m/z 109.0 ($C_6H_5O_2^+$), 123.0 ($C_7H_7O_2^+$), 291.1 ($C_{15}H_{15}O_6^+$), 303.0 ($C_{15}H_{11}O_7^+$), 319.4 ($C_{15}H_{11}O_8^+$), and 333.2 ($C_{16}H_{13}O_5^+$). We also observed the peak at 273.2 ($C_{15}H_{13}O_7^+ - H_2O$). However, only the m/z fragments at 307.4 ($C_{15}H_{15}O_7^-$) and 319.4 ($C_{15}H_{11}O_8^-$) were observed in the negative ion mode. The MS for BT extracted with hexane shows fragments corresponding to CH and m/z 108.0 ($C_6H_4O_2^+$), 109.3 ($C_6H_5O_2^+$), 138.3 ($C_7H_6O_3^+$), and 291.1 $[M + H]^+$ in the positive ion mode, but no GC or ECG fragmentations. Fig. 2 shows the GC, CH, and ECG structures demonstrating possible fragmentation sites.

The GT extracted with methanol in the positive ion mode shows the $[M + H]^+$ ($C_{15}H_{15}O_6^+$, m/z 291.1) for CH and a fragment at m/z 168.2 ($C_8H_8O_4^+$) and 181.0 ($C_9H_9O_4^+$) corresponding to fragments from GC. As with the BT extract, several masses were detected that correspond to ECG. The most essential for ECG in the methanol extract of green tea were the $[M + H]^+$ ion ($C_{22}H_{19}O_{10}^+$, 443.5), and fragments at m/z 123.1

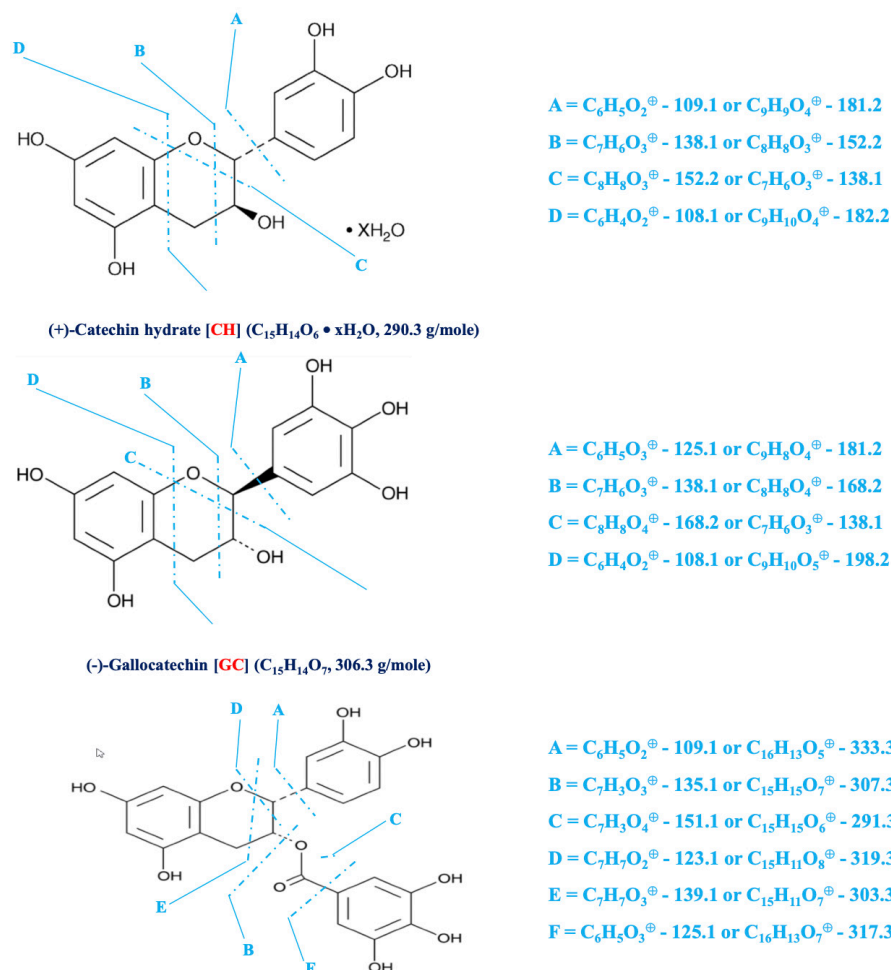


Figure 2. Illustrative fragmentation patterns of catechins detected in BT, GT, and PTT extracts

($C_7H_7O_2^+$), 135.1 ($C_7H_3O_3^+$), 291.1 ($C_{15}H_{15}O_6^+$), 307.3 ($C_{15}H_{15}O_7^+$), and 319.2 ($C_{15}H_{11}O_8^+$). The fragments observed at m/z 273.3 and 289.1 were attributed to fragments at 291.1 ($C_{15}H_{15}O_6^+$ - H_2O) and 307.3 ($C_{15}H_{15}O_7^+$ - H_2O) losing H_2O , respectively¹. The molecular ion occurs at m/z 264.2. The $[M - H]^-$ ion at m/z 441.4 ($C_{22}H_{17}O_{10}^-$) and a fragment at 109.3 ($C_6H_5O_2^-$) were observed in the negative ion mode. None of the negative ion masses matched the masses or fragments for GC, CH, and ECG. In the positive ion mode, the hexane extract of GT shows mass fragments that correspond to CH at m/z 166.2 ($C_9H_{10}O_3^+$). The spectra shows ions for ECG that corresponded to the $[M + H]^+$ ion at m/z 443.0, and fragments at m/z 109.1 ($C_6H_5O_2^+$), 129.9 ($C_7H_7O_2^+$), 134.9 ($C_7H_3O_3^+$), 291.0 ($C_{15}H_{15}O_6^+$), 307.4 ($C_{15}H_{15}O_7^+$), and 333.2 ($C_{16}H_{13}O_5^+$). We also observed the

273.2 ($C_{15}H_{15}O_6^+$ - H_2O) and 289.1 ($C_{15}H_{15}O_7^+$ - H_2O) peaks. The molecular ion was at m/z 701.6, which is an unidentified ion. In the negative ion mode, none of the masses for the hexane extract matched the fragments from either GC or CH. However, the $[M - H]^-$ ion at m/z 441.6 ($C_{22}H_{17}O_{10}^-$) and a fragment at m/z 319.5 ($C_{15}H_{11}O_8^+$) were detected for ECG.

With the PTT sample, several fragments were observed. In the positive ion mode, the most critical masses shown by paradise tropical tea extracted with methanol were the $[M + H]^+$ peak of CH at m/z 291.1 ($C_{15}H_{15}O_6^+$), and fragments at m/z 108.3 ($C_6H_4O_2^+$), m/z 109.4 ($C_6H_5O_2^+$), m/z 138.1 ($C_7H_6O_3^+$), and m/z 181.1 ($C_9H_9O_4^+$). The ECG response observed were the $[M + H]^+$ ion at m/z 443.2, and fragments at m/z 109.1 ($C_6H_5O_2^+$), 135.1 ($C_7H_3O_3^+$), 291.0 ($C_{15}H_{15}O_6^+$),

307.4 ($C_{15}H_{15}O_7^+$), 317.5 ($C_{16}H_{13}O_7^+$), and 319.4 ($C_{15}H_{11}O_8^+$). We also observed the 273.2 ($C_{15}H_{15}O_6^+ - H_2O$), 289.1 ($C_{15}H_{15}O_7^+ - H_2O$), and 299.3 ($C_{16}H_{13}O_7^+ - H_2O$) peaks. The molecular ion on the MS was at m/z 108.3. Two additional masses observed in the positive ion mode spectra matched the $[M + H]^+$ ion ($C_{15}H_{15}O_7^+$, m/z 307.4) and a fragment at m/z 181.0 ($C_9H_9O_4^+$) of GC. None of the masses observed in the negative ion mode matched the masses or fragments of CH. However, ECG showed some fragments starting with m/z 139.0 ($C_7H_7O_3^-$), 371.4 ($C_{16}H_{13}O_7^+$), and 299.4 ($C_{16}H_{13}O_7^+ - H_2O$). With the hexane extract, fragments that correspond to ECG in the positive ion mode were at m/z 125.2 ($C_6H_5O_3^+$, small response), 139.1 ($C_7H_7O_3^+$), 291.2 ($C_{15}H_{15}O_6^+$), 319.4 ($C_{15}H_{11}O_8^+$), 273.2 ($C_{15}H_{15}O_6^+ - H_2O$), and 443.2 ($C_{22}H_{19}O_{10}^+$). The other two significant fragments identified with the hexane extract of PTT were the m/z 139.1 in the positive ion mode

($C_7H_7O_3^+$) and the m/z 125.0 in the negative ion mode ($C_6H_5O_3^-$). These fragments are consistent with catechins in the absence of gallate and have been reported in previous findings^{1,38}. While the spectra were collected in the full scan mode, it thus confirmed CH, GC, and ECG were present in the tea samples analyzed. Fig. 2 shows the structures and fragmentation patterns used in the interpretation. Fig. 3 and 4 show an MS of tea sample extracts extracted with methanol in positive and negative ion modes.

Method validation studies (Agilent 1260 HPLC-DAD)

Table 1 summarizes the calibration responses for the six catechins investigated. The calibration plots enable us to determine slopes, intercepts, correlation coefficient (R^2) values, the limit of detection (LOD), and the limit of quantitation (LOQ). We determine the LOD and LOQ

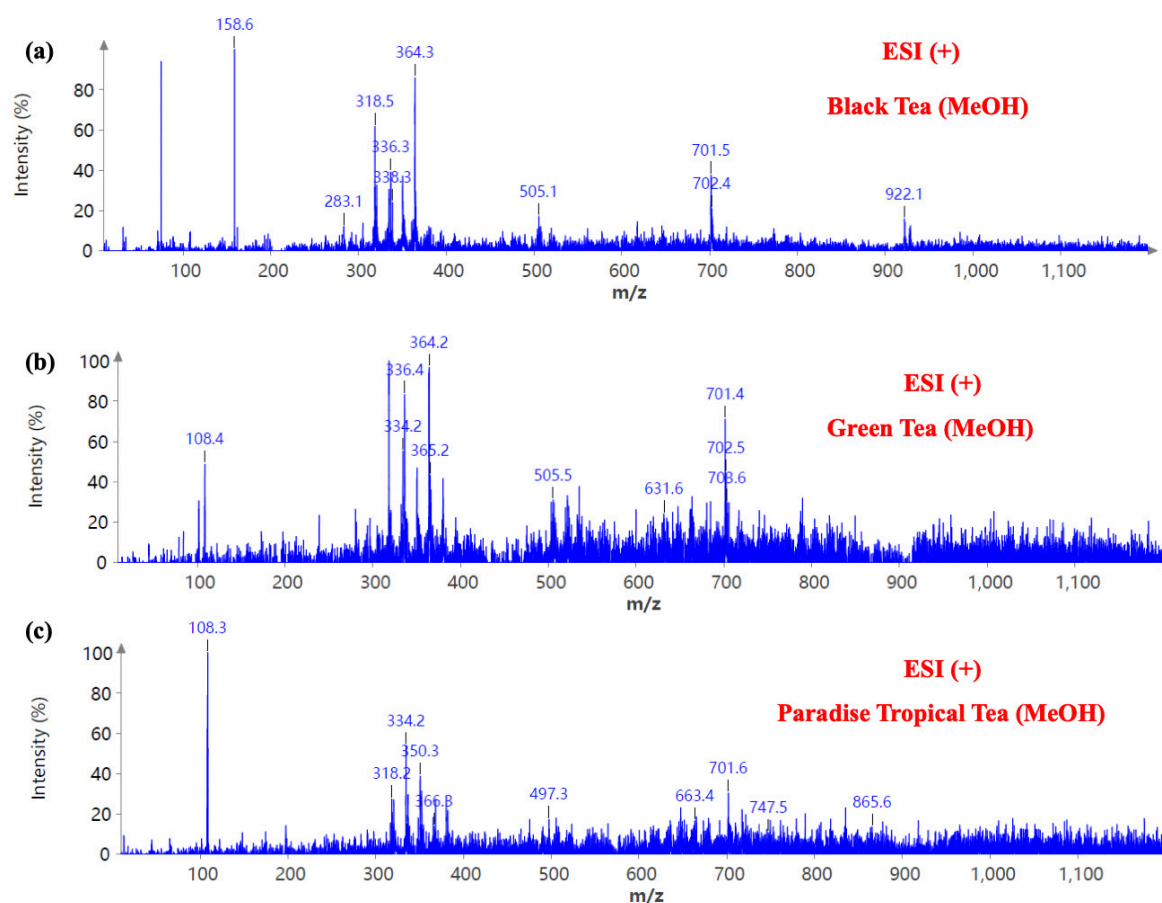


Figure 3. Example MS spectra of BT, GT, and PTT in the positive ion mode

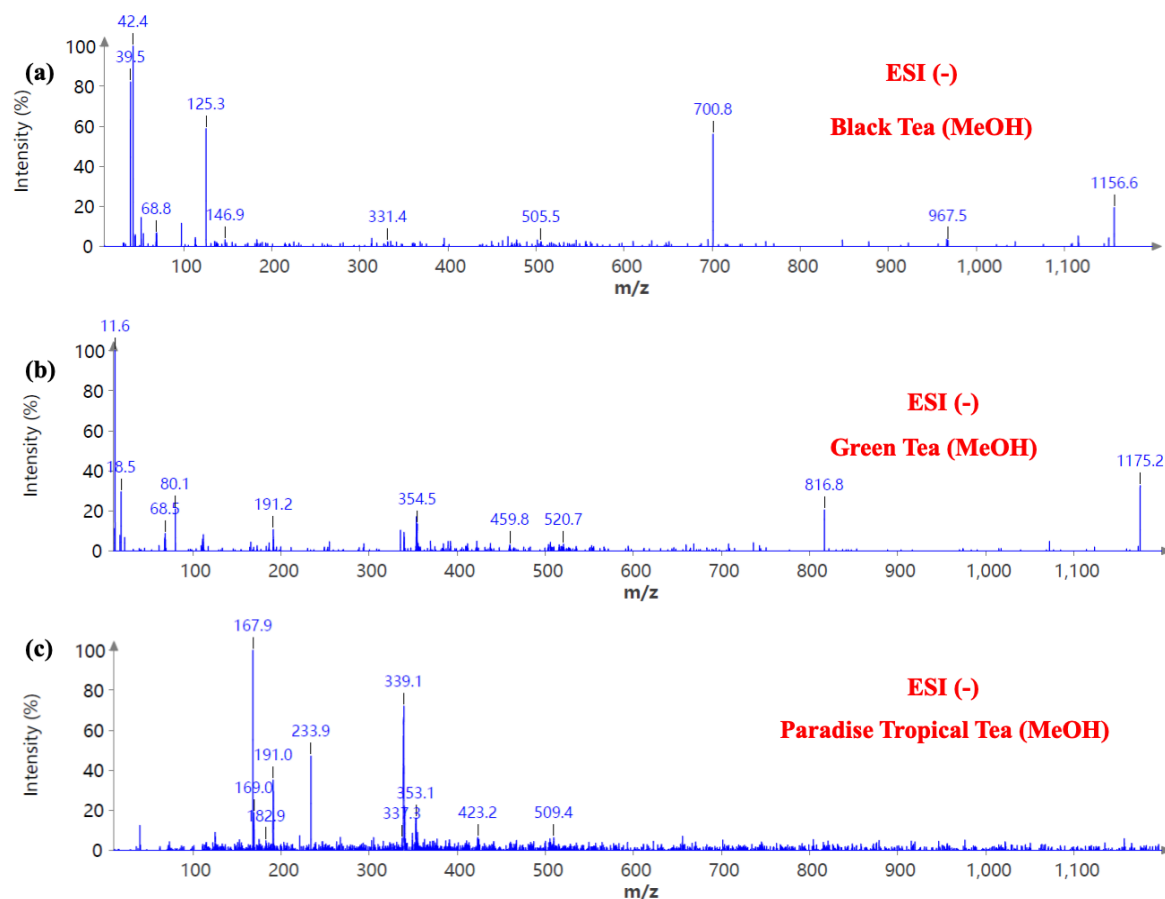


Figure 4. Example MS spectra of BT, GT, and PTT in the negative ion mode

Table 1. Summary of method validation parameters for catechins investigated using the RP-HPLC-DAD

Analyte	B_1/ppm	B_0	R^2	LOD/ppb	LOQ/ppb
Catechin	0.076 ± 0.003	0.005 ± 0.001	1.0000	07.32 ± 0.04	24.39 ± 0.03
Galocatechin	0.021 ± 0.004	0.002 ± 0.001	0.9999	36.44 ± 0.02	121.45 ± 0.16
Epigallocatechin	0.053 ± 0.002	0.022 ± 0.004	0.9995	12.20 ± 0.03	40.68 ± 0.05
Epicatechin gallate	0.056 ± 0.005	0.116 ± 0.011	0.9956	19.73 ± 0.04	65.78 ± 0.01
Galocatechin gallate	0.083 ± 0.002	0.009 ± 0.002	1.0000	05.26 ± 0.02	17.52 ± 0.03
Epigallocatechin gallate hydrate	0.078 ± 0.003	0.042 ± 0.004	0.9994	08.75 ± 0.03	29.15 ± 0.02
The calibration summary for each response is given by the following equation: $\text{Peak area response} = B_0(\text{mAU}) + B_1(\text{mAU} \cdot \text{ppm}^{-1}) \times [\text{concentration}](\text{ppm})$ where B_0 is the intercept and B_1 is the sensitivity or slope					

by injecting replicate runs of the minimum detectable concentration for each catechin. Each of these minimum catechin concentrations was discernable from the instrument noise. The lowest LOD and LOQ were reported for GCG

and GC at 5.26 ± 0.02 ppb and 17.52 ± 0.03 ppb, respectively, with a sensitivity of 0.083 ± 0.002 mAU ppb⁻¹. The highest LOD and LOQ are reported for GCG and GC at 36.44 ± 0.02 ppb and 121.45 ± 0.16 ppb, respectively, with a

Table 2. Standard addition quantitation data for catechins detected in methanol tea extracts using the RP-HPLC-DAD

	Black Tea (mg/g)	Green Tea (mg/g)	Paradise Tropical Tea (mg/g)
Galliccatechin	0.201 ± 0.041	0.354 ± 0.078	3.235 ± 0.175
Catechin hydrate	0.545 ± 0.035	0.511 ± 0.081	4.914 ± 0.266
Epicatechin Gallate	0.241 ± 0.022	0.105 ± 0.051	0.425 ± 0.066

sensitivity of 0.021 ± 0.004 mAU ppb⁻¹. R² values were in the range of 0.9956-1 (see Table 1).

The standard addition calibration method was applied to the catechins detected in the methanol extract of the tea samples. The experimental design is shown in Table S1. The total content for the three catechins detected was 0.996, 0.969, and 8.572 mg/g in BT, GT, and PTT, respectively. The label on the bag of PTT indicated it is made of 100% black tea and natural and artificial tropical fruit flavors. Table 2 is a detailed representation of each catechin detected and quantified in the tea samples.

Conclusions

This work presents an improved HPLC method for determining catechins simultaneously in tea sample extracts using a Luna 3u C₁₈ column. The mass spectra data reveal most of the fragmentation patterns of the three catechins detected in the tea sample extracts. The validation method indicates that the HPLC method is repeatable, reproducible, and sensitive. This method showed a successful validation, and the three catechins can be determined simultaneously in the matrix of the tea extract using the standard addition calibration method. The approach presents several advantages, such as separation, identification, and improved chromatographic efficiency. It further shows the quantitation of catechins in complex mixtures, which is beneficial for analyzing catechins in tea extracts, dietary supplements, and other complex matrices.

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Supplementary information

The complete supplementary information (SI) section has the following information: experimental procedure, Table S1, Table S2, Table S3 and Fig. S1.

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