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Determination of Epicatechins in Tea by Liquid Chromatography – Mass Spectrometry

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Abstract

A sample of brewed *Our Finest* Lemongrass green tea was analyzed by liquid chromatography – mass spectrometry to determine epicatechin content. Epicatechin is a type of catechin, compounds found in fruits and vegetables which have been linked to a number of health benefits. Five epicatechin external standards were prepared along with three replicate brewed tea samples and run on an Agilent Technologies G530 Accurate-Mass Q-TOF LC/MS 1200 series instrument. The MS ionization source was electrospray ionization. The R^2 value of the calibration curve was 0.9763. The concentration of epicatechin in the brewed tea was determined to be 7.88 ppm with a relative standard deviation of 33.8%. The epicatechin content of dry tea was calculated to be 0.751 mg/g. If the experiment was to be done again, an internal standard would be included to potentially improve precision and accuracy.

Introduction

Green tea is one of the oldest and most popular beverages in the world. It is known for its health benefits and can facilitate weight loss, help prevent cancer, and fight certain types of heart disease. The compounds largely responsible for tea's beneficial properties are catechins. There are many different catechins, but they can all be classified as flavonoids, a type of polyphenol found in fruits and vegetables. Catechins themselves have been linked to anti-inflammatory, antioxidant, anti-allergy, and anti-obesity effects in multiple clinical studies.[3, 1]

Because of the beneficial properties of catechins, especially their antioxidant capability, reliable determination of their concentration is important.[1] Antioxidant capabilities differ between catechin isomers, so a specific assay is also important. This experiment focuses on determining (-)-epicatechin in brewed green tea, a matrix with a relatively high concentration of catechins.

Liquid chromatography – mass spectrometry (LC/MS) is an analytical instrument which uses the identification and quantification capabilities of mass spectrometry (MS) to determine analytes separated by liquid chromatography (LC). LC uses a solvent mobile phase to run a sample through a column containing the stationary phase. The sample components are separated based on differing interactions with the stationary phase, with more highly retained species exiting the column later. The eluent is then pumped to a detector – in this case the mass spectrometer – for quantification.

MS works by ionizing molecules in the solution, and then sorting them by mass to charge ratio, or m/z. The most common method of ionization is electrospray ionization or ESI, which disperses the eluent into a mist. The solvent then evaporates and leaves the molecular ions to be accelerated to the mass analyzer.[2] The instrument in this experiment is a quadrupole time-of-flight (Q-TOF) mass analyzer, which differentiates species mass by measuring the time it takes for them to reach the detector.[3] By applying the same amount of kinetic energy to all ions, their speeds will depend solely on their mass. This coupled instrument gives a mass spectrum of relative abundance versus m/z, and also a chromatogram which gives relative abundance (measure in peak area) versus retention time.

Experimental

Standard Preparation Procedure:

To prepare each standard, the appropriate volume of 100 ppm Epicatechin standard solution (see Table 1) was dispensed into a liquid chromatography vial using an Eppendorf pipette. The appropriate volume of citric acid was then added to dilute each standard to a volume of 1.500 mL.

Table 1. Volume of 100 ppm Epicatechin stock solution and citric acid used in preparation of standards.

Standard	Concentration of Epicatechin (ppm)	Volume of 100 ppm epicatechin solution (mL)	Volume of citric acid solution (mL)	Total volume (mL)
S0	0.00	0.000	1.500	1.500
S1	10.0	0.150	1.350	1.500
S2	20.0	0.300	1.200	1.500
S3	30.0	0.450	1.050	1.500
S4	40.0	0.600	0.900	1.500
S5	50.0	0.750	0.750	1.500

Sample Information: *Our Finest/Ingredient Wise* Lemongrass Green Tea

Sample Preparation Procedure:

First, both a full and an emptied teabag were weighed to determine an approximate mass of tea used. The full teabag was then left in boiling 18 MΩ water for 5 minutes. The final volume of brewed tea was 200 mL. A 0.45 µm syringe filter was then used to transfer 1.5 mL of the brewed tea to a liquid chromatography vial. A triplicate of sample vials were prepared.

Analyte Information:

Epicatechin

Molar Mass: 290

Expected m/z: 291 (protonated to +1 cation)

Instrument: Agilent Technologies G530 Accurate-Mass Q-TOF LC/MS 1200 series

Column: Agilent Eclipse plus C18 column

Table 2. Instrument parameters of the LC/MS instrument used for this analysis.

VCap	3500 V
Fragmentor	60
Gas Temperature	350℃
Drying Gas	13 L/min
Nebulizer	60 psig
Sheath Gas Temp	325℃
Sheath Gas Flow	12 L/min
Flow Rate	0.4 mL/min
Injection Size	0.2 µL
Column Temp	50 ℃
Solvent A	74.7% 18 MΩ water, 25% Methanol, 0.3% Formic acid
Solvent B	99.7% Acetonitrile, 0.3% Formic acid
Elution Gradient	100% A to 100% B in 5 mins, hold 5 mins, change to 100% A in 5 mins, hold 5 mins.
Column Internal Diameter	1.8 µm
Column Width x Length	2.1 mm x 100 mm
Ionization Source	Electrospray Ionization (+)
Detector	Diode array

Data and Results

Table 3. Retention time and peak area of the epicatechin peak (found at 291 m/z) in each standard solution.

Standard	Concentration of epicatechin (ppm)	Retention Time (mins)	Peak Area
S0	0	N/A	0
S1	10.0	1.796	6328322
S2	20.0	1.835	13115214
S3	30.0	1.835	16134910
S4	40.0	1.834	25392615
S5	50.0	1.865	26687493

Table 4. Retention time and peak area of the epicatechin peak (found at 291 m/z) in each tea sample.

Sample	Retention Time (mins)	Peak Area
T1	1.888	2365812
T2	1.838	7278280
T3	1.854	5765074
	Average	5136389

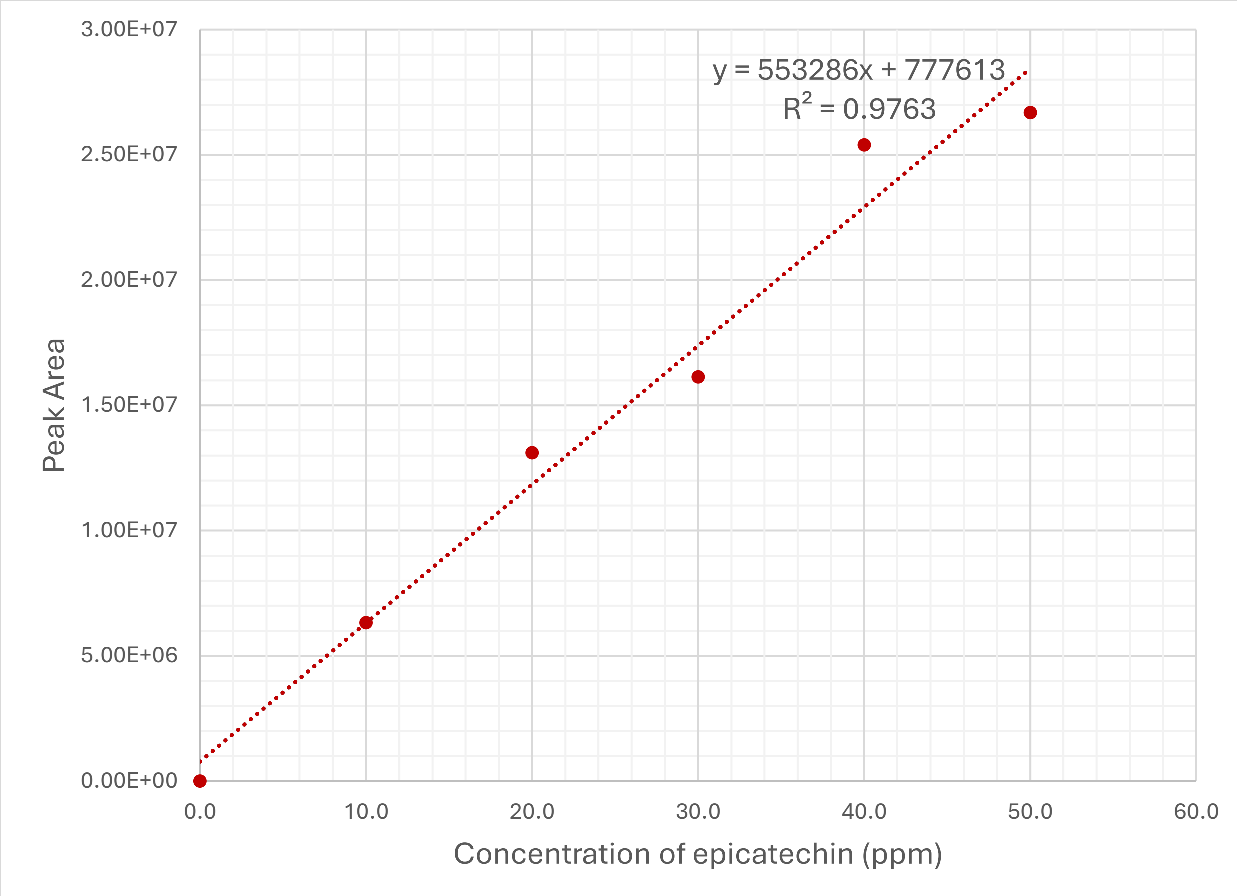


Figure 1. Calibration curve obtained from the peak area of the epicatechin peak at 291 m/z for each standard solution.

Table 5. Calculated results from the determination of epicatechin in green tea.

Literature concentration	7.92 mg / g [4]
Average concentration of epicatechin	7.88 ppm
Amount of tea used	2.099 g
Mass epicatechin in tea*	0.751 mg epicatechin/g dry tea
Uncertainty (s_x)	3.88
%RSD	33.8%
% Error	90.5%
Method of least squares	
Slope	$5.53 \times 10^5 \pm 4.31 \times 10^4$
Intercept	$7.78 \times 10^5 \pm 1.31 \times 10^6$
R^2	0.9763

*For a steep time of 5 minutes in boiling water.

Calculations

Amount of tea used = mass full teabag – mass empty teabag

$$= 2.4179\ g - 0.4080\ g = 2.099\ g$$

Concentration of standard 2:

$$C_2 = \frac{C_1 V_1}{V_2} = \frac{100\ ppm \times 0.300\ mL}{1.500\ mL} = 20.0\ ppm$$

Concentration of epicatechin in tea sample (derived x):

$$\text{Average Peak Height} = \frac{2365812 + 7278280 + 5765074}{3} = 5136389$$

$$\text{Eqn of the line: } y = 553286x + 777613$$

$$Concentration\ (derived\ x) = \frac{5136389 - 777613}{553286} = 6.611$$

Amount of epicatechin in tea:

$$7.88\ ppm = 7.88 mg/L$$

$$7.88 \frac{mg}{L} \times 0.200\ L\ brewed\ tea = 1.576\ mg$$

$$1.576\ mg \times \frac{1}{2.099\ g\ dry\ tea} = 0.751\ mg/g$$

$$= 0.751\ mg \text{ epicatechin per 1 g of dry tea}$$

Uncertainty Calculations (see Appendix 1):

$$S_y^2 = \frac{\sum d_i}{n-2} = 3.25 \times 10^{12}$$

$$D = n\sum x_i^2 - (\sum x_i)^2 = 10500$$

$$S_m = \frac{nS_y^2}{D} = \frac{6 \times 3.25 \times 10^{12}}{10500} = 43110$$

$$S_b = \sqrt{\frac{S_y^2 \times \sum x_i^2}{D}} = \sqrt{\frac{3.25 \times 10^{12} \times 5500}{10500}} = 1.31 \times 10^6$$

$$S_x = \frac{s_y}{|m|}\sqrt{\frac{1}{k}+\frac{1}{n}+\frac{y-\bar{y}_i}{m^2(\sum x_i-\bar{x})^2}} = \frac{1.80\times 10^6}{5.53\times 10^5}\times\sqrt{\frac{1}{3}+\frac{1}{6}+\frac{5.14\times 10^6-1.46\times 10^7}{(5.53\times 10^5)^2\,(150-25)^2}}$$

$$= 2.66$$

Percent RSD:

$$\%RSD = \frac{S_x}{x}\times 100\% = \frac{2.66}{7.88}\times 100\% = 33.8\%$$

Relative error:

$$\frac{true-exp}{true}\times 100\% = \frac{7.92-0.751}{7.92}\times 100\%$$

90.5%

Discussion

The analysis of epicatechin in brewed tea was completed using liquid chromatography coupled to mass spectrometry. The method of external calibration was used and 5 epicatechin standard solutions ranging from 10 to 50 ppm were prepared in citric acid, as well as a blank. The use of citric acid solution in the standards may be to give the standards a pH more similar to green tea, increase solubility of epicatechin, or to prevent epicatechin degradation.

Both liquid chromatography solvents which make up the eluent (see Table 2) contained 0.3% formic acid. The purpose of the acid was to protonate the analyte so that it becomes a cation, and can be detected by the mass spectrometer. The peak of interest is that with a mass to charge ratio (m/z) closest to 291, which is the molar mass of monoprotonated epicatechin. The peak area of this peak was used for determination of epicatechin concentration through generation of an external standard calibration curve.

The mass spectra for all standards and samples had clear peaks at 291 m/z and the chromatogram peaks obtained from the standards (Appendix 2) showed good resolution from other solutes but were not symmetrical. The chromatogram peaks from the tea samples (Appendix 3) showed some interference with another, smaller peak, which could have affected the results. This is an example of the matrix effect of the tea, which could have affected the results in other ways.

The calibration curve in Figure 1 was generated from the chromatograms of the standards, including a point representing the blank. The equation of the line was determined to be $y = 5.53 \times 10^5x + 7.78 \times 10^5$. An R^2 value of 0.9763 was attained, and an uncertainty table (appendix 1) was generated from calibration curve analysis. The relative uncertainty of the intercept (S_b) was 168%, an extremely poor value. The uncertainty in the slope (S_m) is better at about 8% but the curve as a whole is fairly poor quality and reduces the precision of the sample measurement.

Chromatograms and mass spectra were obtained of three replicate brewed green tea samples and the peak areas at m/z = 291 was used to determine epicatechin concentration. The average peak area was 5.14×10^6 and by using the equation of the line, the epicatechin concentration was determined to be 7.88 ppm. A high uncertainty of 2.66 was calculated giving a percent relative standard deviation of 33.8%. This precision is unacceptable and indicates low reproducibility of the method. Notably, the average sample peak area lies below that of the 10 ppm standard, which means it lies near the bottom of the dynamic range. This means unfavourable interpolation is necessary, contributing to poor precision.

A literature value for epicatechin content of dry green tea was found to be 7.92 mg/g[4]. The calculated experimental value for this analysis was 0.751 mg/g, which is about 10 times less than literature, giving a percent relative error of 90.5%. However, this number could be highly variable depending on factors like how long the tea is steeped for or the temperature of the water. If it is the case that epicatechin is soluble enough in water to achieve complete extraction in the 5 minute steep time used for this experiment, then the relative error of this analysis would be more significant. The epicatechin concentration could also vary between brands or type of green tea. Given these factors, and that the experimental and literature value differ by only one order of magnitude, this analysis can be considered at least somewhat accurate.

The precision and reproducibility of this analysis was quite poor, and there are multiple factors that could have contributed. Matrix interference in the brewed tea samples could have caused inconsistencies in the peak of interest of the sample chromatograms. Not using an internal standard approach may have also affected results since external standard calibration alone does not account for matrix interference, sample loss, and instrument variability. Internal standard use is recommended for LC/MS since small variation in ionization efficiency, especially from ESI, is common, along with other instrument variation.[5]

Conclusion

The concentration of epicatechin was determined in brewed *Our Finest* Lemongrass Green Tea to be 7.88 ppm with a relative standard deviation of 33.8%.

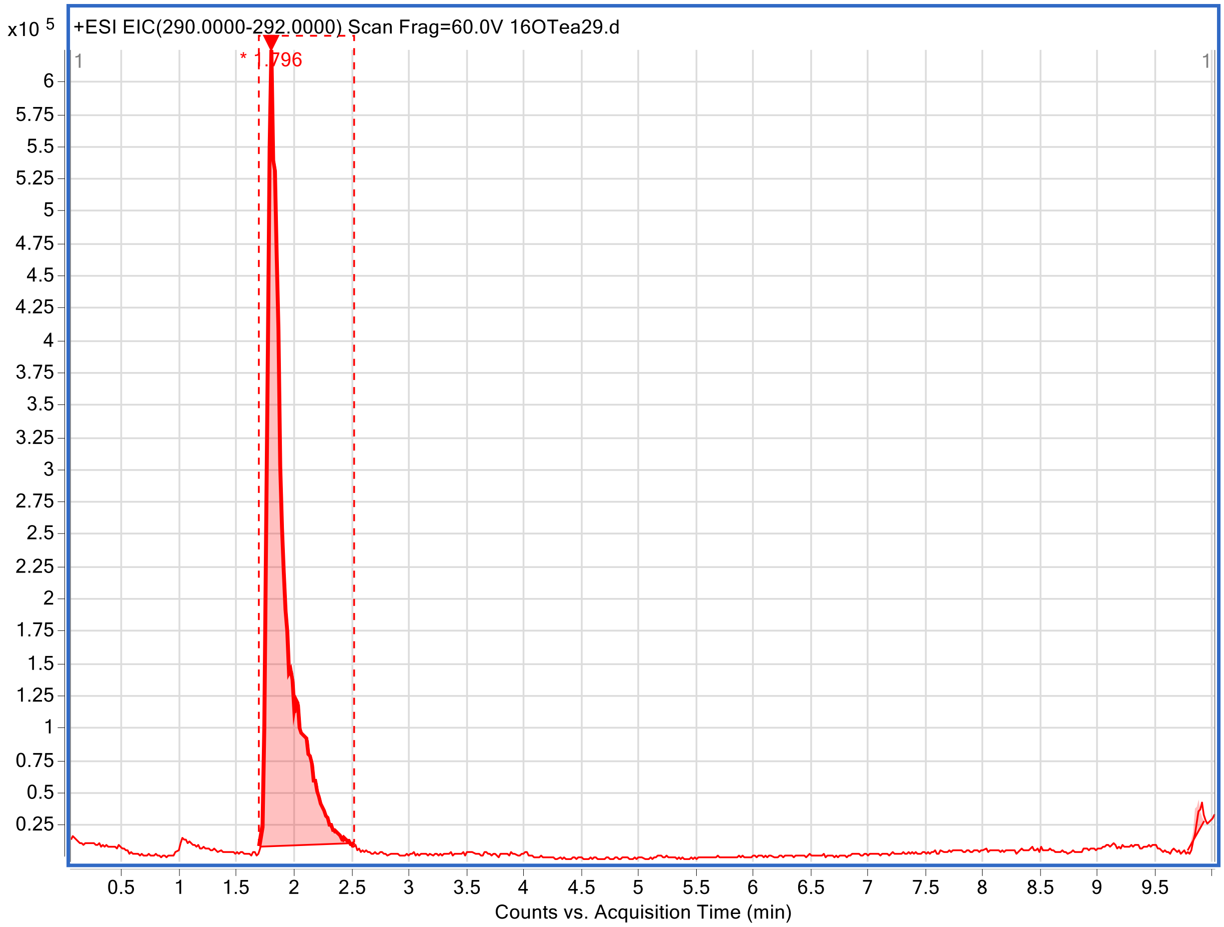
References

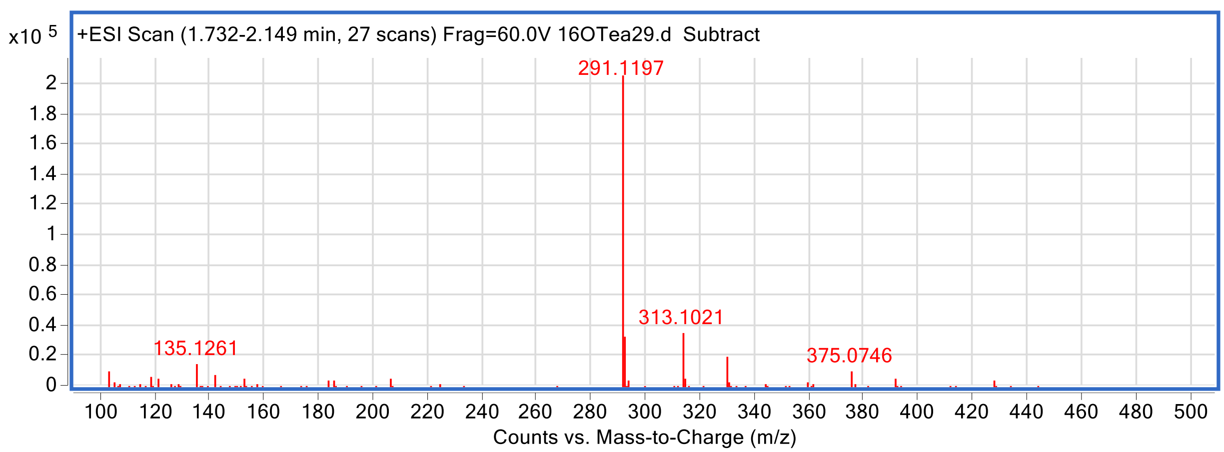
1) Hu, B.; Wang, L.; Zhou, B.; Zhang, X.; Sun, Y.; Ye, H.; Zhao, L.; Hu, Q.; Wang, G.; Zeng, X. Journal of Chromatography A, 2009, 1216, 3223 – 3231.
2) Manisali, I.; Chen, D.; Schneider, B. Trends in Analytical Chemistry, 2006, 25, 243 – 256.
3) Donkor, K. Chemistry 3170 Instrumental Analysis Laboratory Manual, Fall 2024.
4) Ravindranath MH, Saravanan TS, Monteclaro CC, Presser N, Ye X, Selvan SR, Brosman S. Epicatechins Purified from Green Tea (Camellia sinensis) Differentially Suppress Growth of Gender-Dependent Human Cancer Cell Lines. Evid Based Complement Alternat Med. 2006 Jun;3(2):237-47. doi: 10.1093/ecam/nel003.
5) Wang, M., Wang, C., & Han, X. (2017). Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry-What, how and why?. Mass spectrometry reviews, *36*(6), 693–714. https://doi.org/10.1002/mas.21492

Appendix

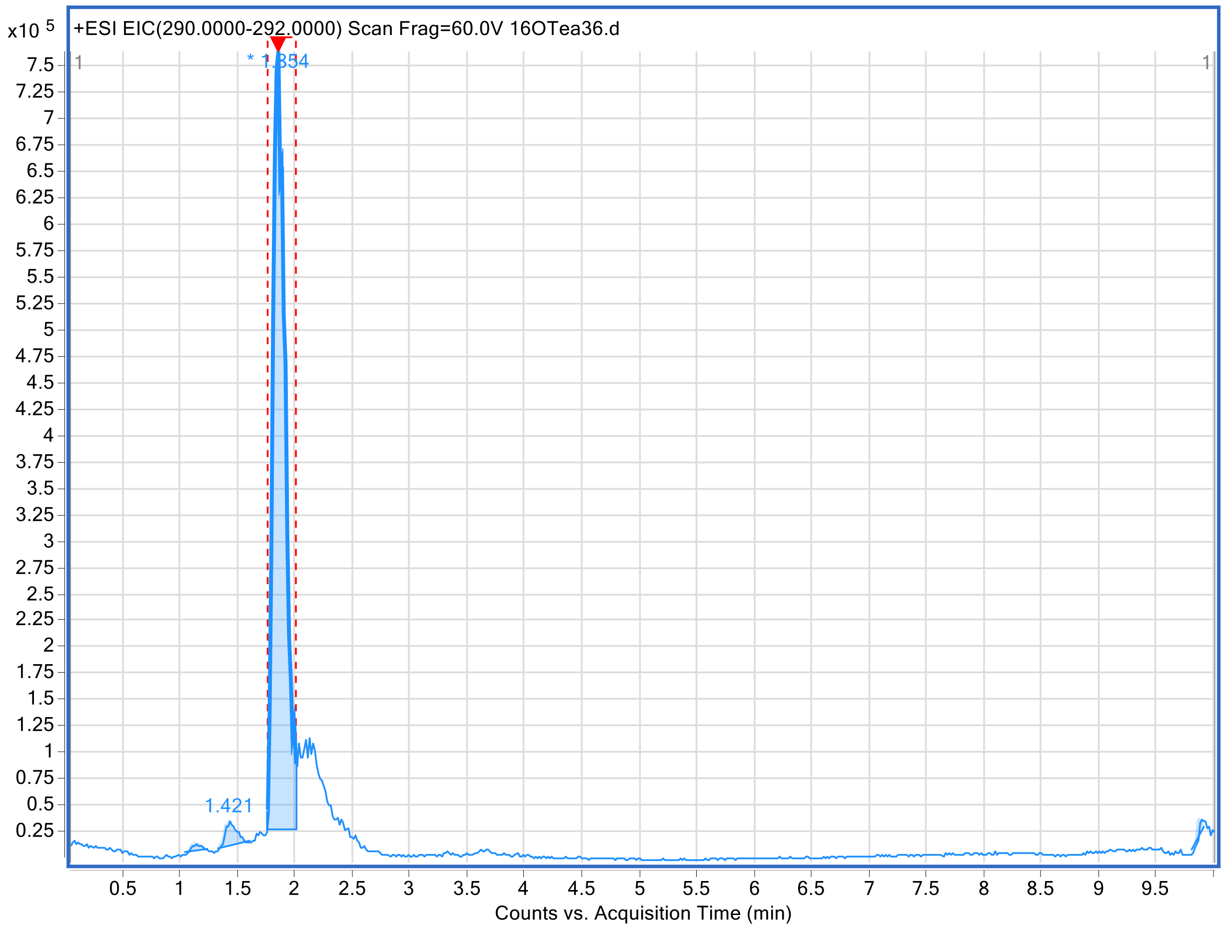
n =	6							
			x_i	y_i	x_iy_i	x_i^2	d_i	d_i^2
			0	0	0	0	-777613	604682422119
Unknowns			10	6328322	63283220	100	17850	318637800
2365812			20	13115214	262304280	400	1271884	1617689272851
7278280			30	16134910	484047300	900	-1241278	1540771427935
5765074			40	25392615	1015704600	1600	2483569	6168112848988
			50	26687493	1334374650	2500	-1754412	3077960463223
		Sums	150	87658554	3159714050	5500		1.30E+13
			D =	10500	S_y =	1.80E+06		
	Method of least squares							
			slope =	5.53E+05	intercept=	7.78E+05		
			R^2 =	0.9763	S_y^2 =	3.25E+12		
			S_m =	43110	S_b =	1.31E+06		
	Measured y value =		5.14E+06		k =	3		
	derived x=	7.88		s_x =	2.66			

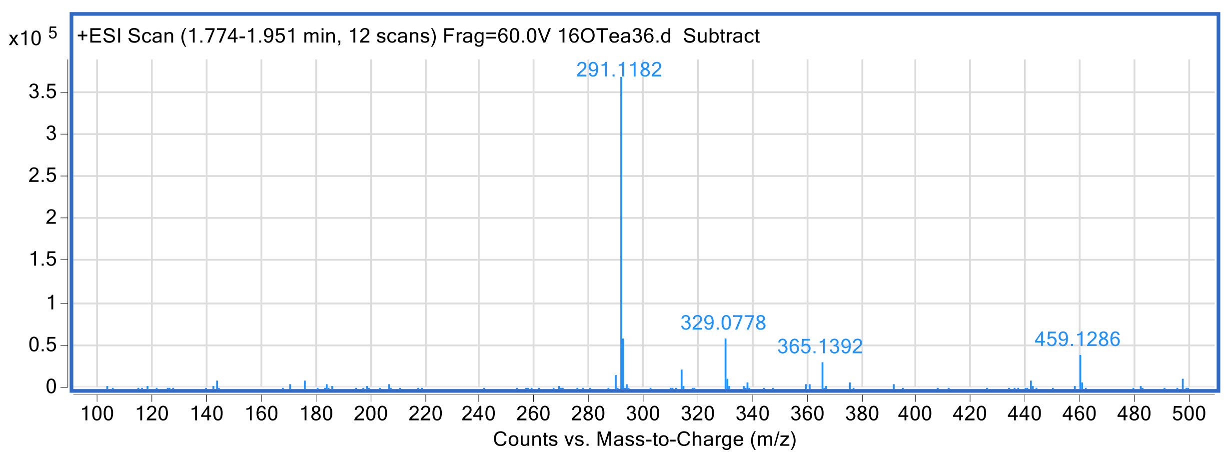
Appendix 1. Uncertainty table for the method of least squares used to generate the calibration curve (Figure 1).





Appendix 2. Chromatogram (top) and mass spectrum (bottom) of Standard 1.





Appendix 3. Chromatogram (top) and mass spectrum (bottom) of tea Sample 3.