The effect of natural antioxidants, pH, and green solvents upon catechins stability during ultrasonic extraction from green tea leaves (*Camellia sinensis*)

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ABSTRACT

Background: This is a first-time report to evaluate the effect of natural antioxidants, pH, and green solvents upon catechins yield and stability during the active process of extraction from green tea leaves.
Methodology: Green solvents (model-A) augmented with piperine (PPN) and quercetin (QT) as natural antioxidants (model-B) at different pH 2–6 (model-C) were used to extract catechins from green tea leaves using an ultrasonic extraction process (USE). For quantification of catechins (EC; epicatechins, ECG; epicatechin gallate, and EGCG; epigallocatechin gallate), a green and sensitive UHPLC-MS/MS method was developed and validated.
Results: The UHPLC-MS/MS method showed an accuracy of 98.3–102.6 % within the linearity range of 1–500 ppb for EC (m/z) $289 \rightarrow 245 \rightarrow 109$, ECG ($m/z$) $441.2 \rightarrow 169 \rightarrow 289$, and EGCG ($m/z$) $457.1 \rightarrow 169 \rightarrow 125.1$. The general yield (ppb) for EC, ECG, and EGCG was observed with the ranges and sum of ($N = 180$) 0.06–157.80 and 6696.83, 0.04–316.93 and 12632.60 and, 0.12–584.11 and 26144.83, respectively. Model-C revealed the highest yield for catechins at the lowest pH-2 with an individual catechin yield of EGCG (584.11) > ECG (316.93) > EC (157.80) in CW2. In terms of stability, EGCG was the most unstable catechin whereas, catechins extracted in model-B exhibited more stability (%recovery of 14.70 for EC, 10.55 for ECG, and 5.36 for EGCG in BEP). Moreover, model-B showed the minimal degradation for catechins within the range of 11.81–94.64 (BEP); even the most degradable EGCG was seen with the smallest %loss of 11.81–94.64 at time 24–70 h, as compared to the loss of > 95 % in model-A and C. The ANOVA score for catechins yield was; $F_{11,168} = 61.06$ (EC), $F_{11,168} = 66.53$ (ECG), and $F_{11,168} = 48.92$ (EGCG) ($P = 0.00$) with mean scores of ($M = 94.63$, $SD = 25.46$) for EC, ($M = 194.87$, $SD = 51.41$) ECG, and ($M = 357.57$, $SD = 96.80$) EGCG in CE2.
Conclusion: A significant effect on catechins yield and stability was observed with the use of natural antioxidants and lowest pH-2.

1. Introduction

Catechins are a class of polyphenols, known as flavonoids, abundantly present in the leaves of the plant *Camellia sinensis* (L.) O. Kuntze, which have been associated with a series of health-promoting effects, including anticarcinogenic, antioxidant, antimicrobial, anti-inflammatory, and anti-thrombogenic activities [1]. Green tea contains higher amounts of catechins than other types of *Camellia sinensis*

teas, primarily because of the post-harvesting process that excludes the fermentation step in green tea compared to other teas, such as black tea [2]. The fermentation of black tea activates an enzyme known as polyphenol oxidase, which catalyzes the oxidation of main catechins into theaflavin, reducing the tea content of catechins [3]. The green tea catechins are also affected by other factors such as origin, growth conditions, harvest process, and preparation and brewing processes. The catechins represent 80–90 % of the green tea flavonoids, and the most

abundant green tea catechins are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) [4]. Of these catechins, EGCG is the most abundant catechin in green tea (~60 %) and has been widely used as a quality indicator and a marker of green tea catechins in kinetic and stability studies.

The quantity of green tea catechins can be easily reduced during extraction as a result of epimerization and degradation. The degradation and stability levels can be primarily influenced by pH, temperature, oxygen availability, the level of metal ions, the presence of antioxidants, and the concentration of catechins and other active ingredients [5]. The temperature is the main factor influencing the conversion of tea catechins to their corresponding isomers, a chemical reaction known as epimerization [6]. This reaction is directly proportional to the temperature and could be observed at 40 °C over prolonged storage. Thus, the epimerization level also depends on the time of storage at high temperatures. The high temperature has been reported to result in the occurrence of simultaneous degradation and epimerization of tea catechins during thermal processes [6]. The degradation of tea catechins is also dependent on the pH level. Tea catechins have been found to be extremely unstable in aqueous solutions when pH is above 6 but very stable at pH lower than 4 [1]. To increase the tea catechins stability, ascorbic acid is commonly used as an antioxidant to decrease oxidative reactions in tea drinks and prevent tea catechins degradation [7]. However, due to the effect of ascorbic acid as a prooxidant, it can enhance the degradation of green tea catechins at storage time longer than one month [8]. This major disadvantage of ascorbic acid necessitates the search for other effective natural antioxidants to enhance the tea catechins stability.

Although previous studies have investigated the effect of antioxidants, pH and temperature on the degradation and stability of tea catechins, there is a lack of studies on the stability of catechins in green organic solvents augmented with unusual natural antioxidants during Ultrasonic assisted extraction (USE). Therefore, this study evaluates the effect of natural antioxidants, pH, and green solvents upon catechins yield and stability during the active extraction process from green tea leaves.

2. Materials and methods

2.1. Solvents, chemicals, and instruments used

The green solvents (Merck; Darmstadt, Germany) of ethanol (EtOH) analytical and LC-MS grade, and acetone (AC) analytical grade were purchased whereas, distilled water (dH$_2$O) was prepared in the lab (Pure Lab; ELGA, High Wycombe, UK). The HPLC-grade pure standard drugs used (Sigma Aldrich; St Louis, MO, U.S.A.) in the study consisted of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), Piperine (97 %), and Quercetin (95 %).

For extraction, Ultrasonicator-(US) (Fisher Scientific; 2000 Park Lane Pittsburgh, PA, USA) was used with the following specifications; power: 50Watt, frequency: 20-kHz, Transducer (Model CL-334), horn (220-A), Titanium probe (420-A; 1 mm diameter), and display power supply. For analysis, Ultra-pressure liquid chromatography-mass spectrometry (UPLC-MS/MS) instrument (UHPLCMS-8050; Shimadzu, Japan) was used with pumps: binary (LC-30AD), column compartment: thermostat controlled (CTO30A), built-in detectors for Uv absorbance: diode-array-detector (DAD; SPD-M20A), and mas quantification: triple-quadrupole with electrospray-ionization-source (TQMS-ESI) as well as a degassing unit, auto-sampler, and instrument controller. LabSolutions (Kyoto, Japan V 5.93) software was used for processing and analysis of the data. For solvent evaporation of the samples, rotavapor (BUCHI Rotavapor®, R-215, Postfach, Flawil, Switzerland) and heating and stirring modules using N$_2$ gas for drying (Thermo Scientific™, Reactitherm III # TS-18824 & 18826, Rockford, IL, USA) were utilized.

2.2. Optimized conditions for ultrasonic assisted extraction (USE)

Herein, our previously developed and validated USE method was applied to extract catechins from the green tea leaves. The effect of different time durations (1–5 min), pulse (20–40 s), duty cycle (5 s), and amplitudes (20–40 %) resulted in an optimum yield at 40 s pulse and 40 % of amplitude using a time duration of 5 min [9]. Hence, the mentioned optimized conditions were applied for catechins extraction in this study. The tea leaves were crushed to a coarse powder, and an amount of 20 mg in 40 mL solvents was used for catechins extraction, as discussed in detail in the forthcoming models.

2.3. Extraction models with evaluation for stability factors

The USE for catechins was processed in three different models of A-C (A, B, and C), where each model consisted of an individual set of extraction factors. The comparative effect upon catechins yield, stability, and degradation was assessed for the different factors applied during the USE of green tea leaves in the three models.

2.3.1. Green solvents extraction of catechins [Model-A]
The first model consisted of two green solvents (EtOH and H$_2$O) only, without adoption of any proposed factor (pH, antioxidants) during the extraction process. Two samples (2 × 1 = 2) for the two green solvents were extracted herein and coded as; AE (ethanol extract in model A) and AW (water extract in model A).

2.3.2. The effect of antioxidants on green solvent extraction of catechins [Model-B]
The model-B tested different antioxidants for their effect upon catechins extraction and their stability during USE. The antioxidants employed in this study consisted of quercetin (QT) and piperine (PPN) at a pre-prepared concentration of (2 % EtOH solution), which were added (2 mL) to the two solvents prior to the actual extraction process. Four samples were prepared for the two antioxidants in two different green solvents of EtOH and H$_2$O (2 × 2 = 4); BEQ (ethanolic extract with QT added in model-B), BEP (ethanolic extract with PPN added in model-B), BWQ (water extract with QT added in model-B), and BWP (water extract with PPN added in model-B). Each sample (20 mg) from green tea leaves was extracted in the mentioned four solvents (40 mL) using USE.

2.3.3. The effect of pH on green solvent extraction of catechins [Model-C]
In model-C, the green solvents at different pH were used during the USE process of green tea leaves in order to evaluate the effect of pH-change upon catechins extraction and stability. Three different pH mediums (2, 6, and 8) were prepared for the two solvents (EtOH and H$_2$O) on an individual basis and USE was carried out for the six samples (2 × 3 = 6) of green tea leaves; CE2 (ethanolic extract at pH-2 in model-C), CE4 (ethanolic extract at pH-4 in model-C), CE6 (ethanolic extract at pH-8 in model-C), CW2 (water extract at pH-2 in model-C), CW4 (water extract at pH-4 in model-C), and CW6 (water extract at pH-6 in model-C). The solvents (40 mL each) were prepared at the desired pH before extraction, and 20 mg of the green tea leaves were added and extracted by USE.

2.4. The stability of catechins in models A-C

The USE-sample for the models A-C were studied for individual catechins stability (EC, ECG, and EGCG) at different time points of 0–94 h (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 70, and 94 h). The time "0 h" refers to an immediate analysis and quantification of catechins in any sample extracted during the process of USE (model A–C), without any delay or further treatments. In order to track the degradation pattern for catechins, an in-house green, fast, accurate, and sensitive method of UHPLC-MS/MS analysis was developed and validated. The equation used to determine the %recovery (stability) for EC, ECG, and EGCG samples is given below:

$$\%recovery = \left(\frac{catechins\ amount\ at\ time\ "94\,h"}{catechins\ amount\ at\ time\ "0\,h"} \right) \times 100$$

2.5. Degradation profile for catechins

Three time points (24, 70, and 94th h) were selected due to the stability of these catechins in the three models till 70 h (EC and ECG), except CE4, CE6, CW2, and CW4 where a degradation of >50 % was seen. For EGCG, a significant degradation initiated after 24 h. Assuming the time "0 h" as 100 % for the catechins data, the %loss was calculated using the formula:

$$\%loss = (catechins\ amount\ "time\ 0\,h"\ (100\%)$$
$$- \%recovery\ calculated\ "time\ 24\ or\ 70\ or\ 90\,h")$$

2.6. Catechins determination using UHPLC-MS/MS

2.6.1. Preparation of catechins standards and stock solutions

The standard solutions (ppb) for individual catechins EC, ECG, and EGCG were prepared in EtOH, and a mix-standard (1 ppb) was prepared by adding the required amount for each standard with a final volume of 1 mL. The stock solution for mix-standard (1 ppb) was diluted further to prepare seven different calibration curve (CC) points in the linearity range of 1–500 ppb (1, 5, 10, 50, 100, 200, and 500 ppb). The samples were syringe-filtered (0.2 μm) and kept in the refrigerator till further use.

2.6.2. Analytical method development for catechins

Nexera X2 UHPLC (Shimadzu, Japan) connected to Shimadzu 8050 triple quadrupole mass spectrometer was used for UHPLC-MS/MS analysis and LabSolutions 5.93 software was used to process the data. The chromatographic separation was performed on an Acquity UHPLC® BEH C18 Column (100 mm, 2.1 mm, 1.7 μm) protected by a guard column (Acquity UHPLC® BEH C18, 1.7 m VanGuard™) from Waters (Ireland). For chromatographic method development, the conditions tested were; the mobile phase consisted of water (A) and ethanol (B), flow rate (0.2–0.4 mL/min), column temperature 40 °C, and sample injection volume of 2–5 μL. For mass analysis of catechins, the condition to run the system were as follows; temperatures of the ESI interface and the desolvation line were 300 °C and 250 °C, respectively. The temperature of the heat block was 400 °C. The flow rate for nitrogen nebulizing gas was 3 L/min whereas, for drying gas and air heating gas the flow was 10 L/min. The UHPLC retention time and the ratio of the two MRM transitions (within 20 % of the ratio in the reference standards) were used to identify the target analytes. When a target analyte was positively identified, it was quantified using the highest intensity MRM transition using external standard calibration.

2.6.3. Method validation for catechins

The developed method was validated in terms of linearity, accuracy, precision, limits of detection (LODs), limits of quantification (LOQs), and matrix effect (ME).

Two MRM transitions were recorded for each analyte, and their specific ratios were calculated for target analyte identification. To confirm the positive detection of an analyte, a retention time (10 % around the mean value) and a ratio between the two MRM transitions with a deviation of no>20 % from the mean value were required.

External standard calibration was used to evaluate the linearity range. Each compound was analyzed in triplicate at six different concentration levels in the range of 1–500 ppb, and the determination of coefficients were calculated.

The extraction recovery was estimated by spiking blank samples at low, medium and high concentration levels (5, 150 and 300 ppb) with six replicates to verify recovery percentages. Recovery was calculated by dividing the peak areas of the target analytes in pre-extraction spiked

samples by post-extraction spiked samples and multiplying by 100. Blank samples were analyzed, and in case any of the target analytes were found, their concentration was calculated and subtracted from the spiked samples. Intra-day precision was evaluated as the %RSD of six replicates (n = 6) measurements performed on the same day. Inter-day precision was evaluated by performing three distinct experiments over three consecutive days, and the results were calculated as the %RSD of these measurements (n = 3 × 3).

Limits of detections (LODs) and quantifications (LOQs) were determined using calibration regression data using the following equations.

$$LOD = 3.3 \times \frac{\sigma}{s} \tag{1}$$

$$LOQ = 10 \times \frac{\sigma}{s} \tag{2}$$

where, σ is the standard deviation of the intercept and s is the regression slope.

Matrix effect (ME) was evaluated using the post-extraction addition method. Samples set A was prepared by extracting blank samples, then spiking standard solution right before injection into the UHPLCMS/MS. Samples set B was prepared by spiking the same analytes concentrations into ultrapure water and injected into UHPLCMS/MS. ME was calculated suing the following equation.

$$ME\ \% = \left(\frac{(Peak\ Area)_{Set\ A} - (Peak\ Area)_{Set\ B}}{(Peak\ Area)_{Set\ B}} \right) \times 100$$

If ME (%) value is 0 %, this means there is no matrix effect. Positive values indicate ion enhancement; however, negative values indicate ion suppression.

2.6.4. Preparation and analysis of USE samples

Any USE sample was filtered (0.2 μm), and prepared for UHPLC-MS/MS analysis. This was termed as "time 0 h" analysis, and the amount of catechins (EC, ECG, EGCG) quantified through the developed and validated UHPLC-MS/MS method was noted. The stability and degradation profile for individual catechins started henceforth, as discussed in the respective sections.

3. Results

3.1. UHPLC-MS/MS method development and validation (MDMV)

The MDMV resulted in a mobile phase consisting of 0.1 % formic acid in ultrapure water (A) and EtOH (B) in gradient elution mode, flow rate of 300 μL/min, sample injection volume of 2 μL, and column temperature at 40 °C. The gradient condition for the developed chromatographic method is presented in Table 1. The run time for catechins MDMV, including the re-equilibration, was 9 min, as shown in the chromatogram (Fig. 1). For mass analysis, ESI (electrospray ionization source) was operated in negative mode and the quantification was achieved using multiple reaction monitoring (MRM). The flow injection analysis (FIA) and the automated MRM optimization technique in LabSolutions® were used to optimize MRM transitions for each analyte. The optimized MRM-parameters for each analyte (EC, ECG, EGCG) are shown in Table 1 whereas, the characteristic values for UHPLC-MS/MS method validation including LODs, LOQs, matrix effect, linearity range, correlation coefficient, accuracy, and precision are shown in Table 2.

3.2. General yield for catechins

The descriptive statistics for sum, mean (±SD; standard deviation), and ranges were computed for individual catechins (EC, ECG, EGCG) in the three models (A-C). The yield (ppb) for EC ($N = 180$) in the three models was observed in the range of 0.06–157.80 with a sum of 6696.83 and a mean ± SD value of 37.20 (35.72). ECG was observed in the range

Table 1

Chromatographic conditions and MS-optimization parameters for catechins MDMV.

Gradient system for Catechins UHPLC-MS/MS MDMV			
Mobile phase	A (0.1 % formic acid in ultrapure water); B (EtOH)		
HPLC	Time (min)	% A	% B
Gradient	0.0	90	10
	1.0	90	10
	3.0	70	30
	4.0	50	50
	4.5	0	100
	5.0	90	10
	7.5	90	10
	Column oven temperature: 40 °C, Flow rate: 0.3 mL/min		

MS/MS optimized parameters and retention times for target analytes								
	Compound	Retention time (min)	Polarity	Precursor ion	Product ion-1 (m/z)[1]	Collision energy (eV)	Product ion-2 (m/z)	Collision energy (eV)
1	EGCG	3.1	–	457.1	169.0	19	125.1	38
2	EC	3.4	–	289.0	245.0	16	109.1	26
3	ECG	4.0	–	441.2	169.0	21	289.0	19

[1]Precursor ion-1 used for quantification.

of 0.04–316.90 with a sum and mean ± SD values of 12632.60 and 70.18 (70.34) whereas, EGCG was seen in the range of 0.12–584.11 with a sum 26144.83 and a mean ± SD values of 145.25 (129.14).

In general, EGCG was more abundant in these samples compared to EC and ECG with a descending order of EGCG > ECG > EC.

3.3. The effect of factorial designs upon catechins yield

3.3.1. Yield in green solvents

The yield for EC in AE (ethanolic solution) showed a range of 3.35–57.11 ppb whereas, AW (aqueous solution) was observed in the range of 0.21–8.29. The sum and mean ± SD values observed were 754.10 and 50.27 (13.47) for AE and, 104.52 and 6.97 (1.91) for AW (Table 3). The range for ECG in the two solvents was 5.18–102.78 and 0.14–19.34 with a sum and mean ± SD values of 1334.98 and 89.00 (24.28) for AE whereas, 258.33 and 17.22 (4.76) for AW, respectively (Table 4). The EGCG exhibited the range of 2.93–208.55 for AE and 1.79–95.10 for AW. Furthermore, 2547.32 and 169.82 (59.39) for AE whereas, 1148.87 and 76.59 (25.239) for AW were observed to be the sum and mean ± SD values, respectively (Table 5).

More yield for EC, ECG, and EGCG in model-A was observed in EtOH (AE extract), with a descending order as; AE > AW whereas, the individual yield for catechins was observed with a descending order of; EGCG (208.55) > ECG (102.78) > EC (57.11) in AE.

3.3.2. Yield in green solvents added with antioxidants

The second model used the natural antioxidants of QT and PPN which were added to the green solvents for catechins extraction. For EC, more yield (57.48 ppb) was observed in BEP with the lowest yield in BWP (8.67 ppb). The ranges for BEP and BWP 8.45–57.48 and 1.01–8.67 were noted with sum and mean ± SD values of 751.01 and 50.07 (±12.20) and, 115.83 and 7.72(±1.93), respectively. The ranges with sum and mean ± SD values for BEQ and BWQ are given in Table 3. Alike EC, ECG showed more yield in BEP (102.25 ppb) in the range of 10.79–102.25 ppb. The lowest yield was observed for BWP (17.73 ppb) within the range of 1.26–17.73 ppb. The sum and mean ± SD values observed were; BEP (1317.41 and 87.83(±22.76)) and BWP (226.09 and 15.07 (±4.18)), as shown in Table 4. EGCG exhibited the same pattern of more yield in BEP (230.20 ppb) with a range (12.34–230.20 ppb), sum (2920.25 ppb), and mean ± SD value of 194.68(±69.78). The lowest yield for EGCG was seen in BWP (91.52 ppb) with a range (4.75–91.52 ppb), sum (940.24 ppb), and mean ± SD value of 62.68(±25.47). The data for EGCG is given in Table 5.

Model-B revealed more yield for EC, ECG, and EGCG in BEP with a

descending order of BEP > BEQ > BWQ > BWP. For individual yield of catechins in model-B, the descending order observed was; EGCG (230.20) > ECG (102.25) > EC (57.48) in BEP.

3.3.3. Yield in green solvents at different pH values

The change in pH-medium (2–6) during USE for catechins exhibited a unique pattern where all the catechins exhibited the highest yield among the three models A-C. More yield was observed at CE2 (low pH-2 in aqueous medium) followed by EtOH at pH-2 whereas, the lowest yield was observed at CE6 (pH-6 in EtOH) for EC and ECG, and in CW6 (pH-6) for EGCG. The highest yields observed for these catechins were; CW2 (157.80 ppb) > CE2 (110.11 ppb) for EC, CW2 (316.93 ppb) > CE2 (220.51 ppb) for ECG and, CW2 (584.11 ppb) > CE2 (439.03 ppb) for EGCG. The lowest yield for EC (0.06 ppb) and ECG (0.06 ppb) was found in CE6; however, for EGCG the lowest yield was seen in CW6 (0.12 ppb). The ranges, sum, and mean ± SD values for EC, ECG, and EGCG at different pH (2–6) are given in detail in Tables 3–5.

Model-C exhibited the highest yield at pH-2 for EC and ECG with a descending order of yield as; CW2 > CE2 > CE4 > CW6 > CW4 > CE6. Though EGCG was observed with highest yield at pH-2, the order for EGCG yield was different; CW2 > CE2 > CE4 > CW4 > CE6 > CW6. With regard to individual catechins yield (ppb) at different pH (2–6) the descending order may be; EGCG (584.11) > ECG (316.93) > EC (157.80) in CW2.

3.4. Stability of catechins

The stability for catechins was determined in terms of individual recovery for all the samples in the three models A–C. The comprehensive representation of stability data is shown in Fig. 2. The descriptive statistics for %recovery revealed a range of 2.53–14.70 for EC, 0.15–10.55 for ECG, and 0.46–5.36 for EGCG. The individual %recoveries (stability) for EC, ECG, and EGCG are discussed in detail below.

3.4.1. Stability in green solvents

In model-A, GS (green solvents only) showed a recovery of 5.87 % in AE followed by AW (2.53 %) for EC. The ECG too, exhibited a recovery of 5.04 % in AE followed by AW (0.74). The EGCG exhibited the least recovery among catechins with 1.89 % in AW whereas, 1.41 in AE. The order for catechins stability in GS was: AE (EC, ECG) > AW (EC, ECG).

3.4.2. Stability in green solvents with added antioxidants

Model-B (GS with AA) showed the highest recoveries for all the catechins; a recovery of 14.70 % (BEP) followed by 11.63 % (BWP) for
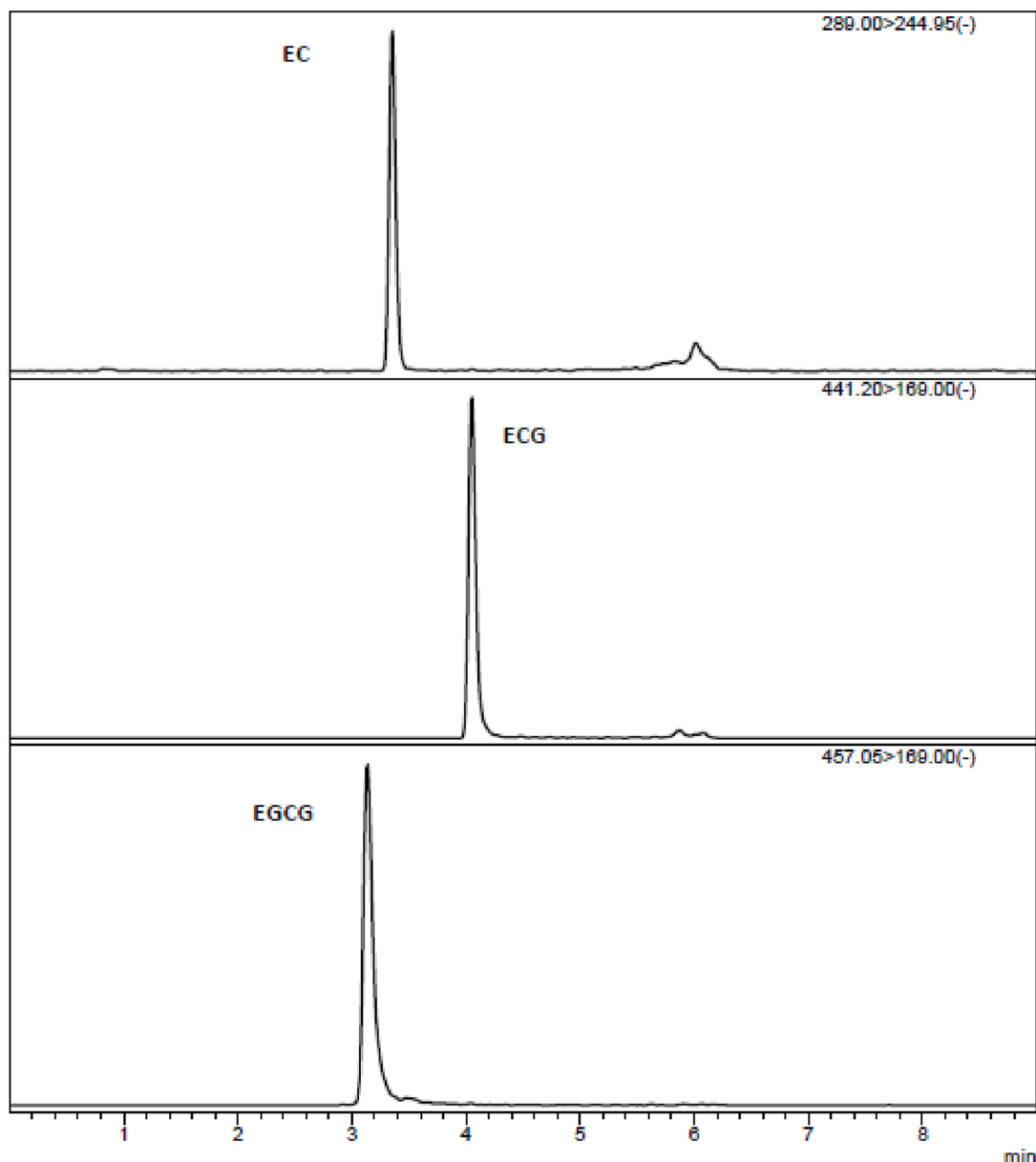
Fig. 1. Representative UHPLCMS/MS chromatogram for EC, ECG, and EGCG separation with *m/z*.

EC, 10.55 % (BEP) and 7.12 (BWP) for ECG and, 5.36 % (BEP) and 5.19 % (BWP) for EGCG. Though BEQ and BWQ exhibited a considerable recovery for EC and ECG when compared to GS model, the recovery for EGCG was less in BEQ. The stability for catechins in model-B was: BEP (EC, ECG, EGCG) > BEQ (EC, ECG, EGCG) > BWQ (EC, ECG, EGCG) > BEQ (EC, ECG).

3.4.3. Stability in green solvents at different pH values

Model-C (GS at different pH) showed a recovery of 7.61 % and 6.88 % for EC in CE6 and CE4, respectively. The recoveries for ECG and EGCG were found more in CW2 and CE2 but with a different order of: CE2 (5.74 %) > CW2 (5.01 %) for ECG and CW2 (4.71 %) > CE2 (4.40 %) for EGCG. EC exhibited a comparatively low recovery in CE2 and CW2-CW6 whereas, ECG and EGCG showed less recoveries in CE4, CE6, CW4, and CW6. The order for stability n model-C was: CE6 followed by CE4 (EC) > CE2 followed by CW2 (ECG) > CW2 followed by CE2 (EGCG).

The data for the three models A-C suggested a general order for catechins stability to be: AA (BEP, BEQ) > pH-2 (CE2 and CW2) > GS (AE). For individual catechins, EC was more stable in BEP > BWP > CE6 > CE4 > AE, ECG in BEP > BWP > CE2 > AE > CW2 whereas, EGCG showed a considerable stability in BEP > BWP > CW2 > CE2 (Fig. 2). The stability order for catechins was: EC > ECG > EGCG.

3.5. Degradation (loss on storage) for catechins

For EC, more degradation was observed in AW (97.5 %) followed by CW4 (96.7 %), CW2 (96.3 %), CW6 (95.3 %), CE2 (95.2 %) and so forth whereas, BEP (85.3 %) and BWP (88.4 %) revealed a comparatively less degradation among the three models A-C. For ECG, more degradation was seen in CW4 (99.8 %), AW (99.3 %), CE6 (97 %), and CE4 (96.4 %) with a lesser degradation in BEP (89.4 %) and BWP (92.9 %). EGCG showed the highest degradation with > 95 % in almost all the models except BEP and BWP where a slightly lower degradation of 94.6 and 94.8 % was observed, respectively. The degradation data for catechins is shown in Table 6 and Fig. 3. The degradation order for these catechins was EGCG > ECG > EC whereas, the degradation profile (%loss) for all the samples showed an order with a range of: CW4 (7.56–99.85) > CE4 (49.81–99.54) > AW (6.38–99.26) > BEQ (9.57–98.72) > AE

Table 2

Characteristic method performance parameters of the proposed method ([a] Limit of detection; [b] Limit of quantification); * Accuracy and precision were evaluated at three concentration levels (5, 150 and 300 ng mL^{-1} for the low, medium and high levels, respectively).

Parameters	EC	ECG	EGCG
Calibration range (ppb)	1–500	1–500	1–500
Regression equation			
Slope (b)	3.25×10^3	5.39×10^3	3.90×10^3
Intercept (a)	4.45×10^3	-3.22×10^3	-1.11×10^3
Correlation coefficient (r^2)	0.9993	0.9997	0.9998
Standard deviation of slope (S_b)	38.9	39.1	25.6
Standard deviation of intercept (S_a)	40.8	40.7	26.6
LOD[a] (ppb)	0.04	0.02	0.02
LOQ[b] (ppb)	0.13	0.08	0.07
Matrix effect	−3.5	−4.4	−6.2
Accuracy (%)			
Low	102.2	98.9	99.2
Medium	101.7	100.6	99.2
High	102.6	98.3	101.5
Precision			
Inter-day precision*			
Low	1.3	1.2	1.1
Medium	0.9	0.8	0.6
High	1.1	1.3	1.2
Intra-day precision*			
Low	1.5	0.9	1.5
Medium	1.2	1.1	0.9
High	2.1	1.6	1.3

(8.18–98.59) > CE6 (50.52–96.98) > CW2 (57.64–96.30) > CW6 (13.25–96.04) > CE2 (11.99–95.67) > BWQ (6.52–95.27) > BWP (8.43–94.81) > BEP (11.81–94.64). The descending order for catechins degradation in the three models A-C was: AA (BEP, BWP) < pH (CE2) < GS (AE).

3.6. Statistical models

3.6.1. Descriptive analysis

The descriptive statistics for catechins yield, stability, and degradation data are discussed under the individual respective sections. For the general yields at different time points, the data (low–high range, sum, mean with SD) is shown in tables for EC (Table 3), ECG (Table 4), and EGCG (Table 5).

3.7. One-way ANOVA

3.7.1. Yields in models A–C

The ANOVA results for catechins (EC, ECG, EGCG) exhibited a significant difference in terms of yield with $F_{11,168} = 61.06$ for EC, $F_{11,168} = 66.53$ for ECG, and $F_{11,168} = 48.92$ for EGCG ($P = 0.00$). For individual differences between the groups, post-hoc Tukey's test was performed at $P = 0.05$. The post-hoc analysis revealed significant differences for the catechins mean scores: CE2 ($M = 94.63$, $SD = 25.46$) and CW2 ($M = 87.71$, $SD = 38.95$) for EC, CE2 ($M = 194.87$, $SD = 51.41$) and CW2 ($M = 188.64$, $SD = 76.76$) for ECG, and CE2 ($M = 357.57$, $SD = 96.80$) and CW2 ($M = 350.36$, $SD = 142.64$) for EGCG. The one-way ANOVA data for catechins yield is shown in Table 7.

3.7.2. Catechins degradation

The one-way ANOVA for the degradation profile in model A-C suggested a significant difference for the degradation pattern of catechins ($P = 0.05$) except CE4 and CE6. The ANOVA score for CE4 ($F2,6 = 3.447$, $P = 0.101$) and CE6 ($F2,6 = 1.837$, $P = 0.239$) revealed a non-significant degradation pattern, as shown in Table 8.

4. Discussion

The degradation pattern and stability profile for catechins have been reported in the literature wherein the effect of temperature, humidity, time, presence of metals, and antioxidants have been evaluated. These catechins have been found to undergo degradation, epimerization, polymerization, thermal instability, and oxidation based on the nature of the environment provided during its processing, mainly affected by the change in temperature and pH, heating time, oxygen concentration

Table 3

Data for EC yield (ppb) with descriptive statistics analysis in the three models A-C.

Model	Green solvents only		Green solvents + antioxidants			Green solvents + pH (2–6)						
	A		B			C						
Time (h)	AE	AW	BEQ	BEP	BWQ	BWP	CE2	CE4	CE6	CW2	CW4	CW6
0	57.11	8.29	57.29	57.48	11.06	8.67	110.11	105.30	0.72	157.80	11.80	16.36
2	56.91	8.03	56.93	57.43	11.05	8.54	106.04	95.80	0.41	139.86	11.71	16.18
4	55.60	7.88	56.45	57.30	10.95	8.49	105.75	85.13	0.41	124.45	11.65	16.14
6	55.49	7.73	55.89	57.12	10.77	8.48	104.67	76.92	0.41	113.38	11.63	15.94
8	55.06	7.53	55.66	56.44	10.75	8.46	103.73	75.25	0.39	105.85	11.52	15.93
10	54.86	7.45	55.35	55.24	10.74	8.44	103.17	68.34	0.38	98.89	11.49	15.81
12	54.58	7.40	54.00	55.16	10.69	8.36	102.24	65.64	0.38	93.94	11.49	15.67
14	54.00	7.38	53.84	53.06	10.66	8.31	101.09	63.77	0.34	89.11	11.48	15.59
16	53.59	7.37	52.97	52.63	10.58	8.28	100.65	59.46	0.33	83.71	11.40	15.44
18	53.43	7.29	52.72	51.09	10.35	8.22	100.23	58.57	0.31	78.11	11.29	15.31
20	53.42	7.17	50.09	48.12	10.17	8.15	100.12	56.80	0.31	63.73	11.29	15.26
22	52.73	7.10	48.57	47.90	10.02	7.95	99.98	53.37	0.27	61.63	11.11	15.21
24	52.44	6.92	48.12	47.47	9.95	7.94	93.09	52.85	0.26	57.79	10.91	14.19
70	41.55	6.76	45.00	46.13	8.72	6.52	83.49	36.91	0.25	41.56	9.24	11.79
94	3.35	0.21	3.42	8.45	0.60	1.01	5.33	7.24	0.06	5.84	0.39	0.77
Descriptive statistics												
Mean	*50.27*	*6.97*	*49.75*	*50.07*	*9.80*	*7.72*	*94.64*	*64.09*	*0.35*	*87.71*	*10.56*	*14.37*
Sum	*754.10*	*104.52*	*746.30*	*751.01*	*147.07*	*115.83*	*1419.67*	*961.35*	*5.22*	*1315.65*	*158.41*	*215.57*
SD	*13.47*	*1.91*	*13.32*	*12.20*	*2.61*	*1.93*	*25.46*	*23.56*	*0.14*	*38.94*	*2.88*	*3.93*
Minimum	*3.35*	*0.21*	*3.42*	*8.45*	*0.60*	*1.01*	*5.33*	*7.24*	*0.06*	*5.84*	*0.39*	*0.77*
Maximum	*57.11*	*8.29*	*57.29*	*57.48*	*11.06*	*8.67*	*110.11*	*105.30*	*0.72*	*157.80*	*11.80*	*16.36*

Table 4
Data for ECG yield (ppb) with descriptive statistics analysis in the three models A-C.

Model	Green solvents only		Green solvents + antioxidants				Green solvents + pH (2–6)					
	A		B				C					
Time (h)	AE	AW	BEQ	BEP	BWQ	BWP	CE2	CE4	CE6	CW2	CW4	CW6
0	102.78	19.34	93.33	102.25	34.36	17.73	220.51	161.67	2.02	316.93	23.95	24.32
2	99.52	19.20	92.63	102.20	34.17	17.51	219.35	147.61	0.92	287.77	23.88	23.61
4	98.90	18.91	90.54	101.85	34.11	17.37	217.29	131.60	0.70	255.64	23.85	23.41
6	98.64	18.70	90.32	101.18	33.97	17.23	214.67	117.73	0.47	241.50	23.65	23.12
8	98.17	18.68	89.65	97.98	33.84	16.81	213.07	112.98	0.44	231.54	23.56	22.82
10	97.93	18.55	89.54	97.38	33.72	16.57	209.74	102.32	0.39	217.21	23.33	22.63
12	97.81	18.44	87.49	96.47	33.70	16.53	208.11	94.70	0.36	201.41	23.23	22.57
14	95.16	18.42	87.42	96.44	33.58	16.49	207.56	94.02	0.26	198.45	23.14	21.79
16	94.99	18.33	86.82	91.66	33.50	16.09	207.36	88.59	0.24	185.50	23.10	21.76
18	94.96	18.29	86.76	89.77	33.43	15.89	207.22	82.33	0.23	171.52	22.85	21.49
20	94.77	18.28	85.96	86.95	32.76	15.87	206.88	81.79	0.20	136.31	22.61	21.38
22	93.11	18.18	84.32	82.96	32.53	15.21	204.09	76.06	0.20	133.53	22.50	21.30
24	92.14	18.11	83.04	81.96	32.12	14.88	194.08	73.61	0.10	128.10	21.90	19.48
70	70.93	16.74	71.53	77.57	29.66	10.65	180.44	43.87	0.10	108.43	19.81	16.49
94	5.18	0.14	4.43	10.79	1.79	1.26	12.65	5.88	0.06	15.88	0.04	1.14
Descriptive statistics												
Mean	*89.00*	*17.22*	*81.58*	*87.83*	*31.15*	*15.07*	*194.87*	*94.32*	*0.45*	*188.65*	*21.43*	*20.49*
Sum	*1334.98*	*258.33*	*1223.76*	*1317.41*	*467.24*	*226.09*	*2923.03*	*1414.77*	*6.69*	*2829.72*	*321.38*	*307.31*
SD	*24.28*	*4.76*	*21.96*	*22.76*	*8.21*	*4.18*	*51.40*	*38.90*	*0.49*	*76.77*	*6.01*	*5.68*
Minimum	*5.18*	*0.14*	*4.43*	*10.79*	*1.79*	*1.26*	*12.65*	*5.88*	*0.06*	*15.88*	*0.04*	*1.14*
Maximum	*102.78*	*19.34*	*93.33*	*102.25*	*34.36*	*17.73*	*220.51*	*161.67*	*2.02*	*316.93*	*23.95*	*24.32*

Table 5
Data for EGCG yield (ppb) with descriptive statistics analysis in the three models A-C.

Model	Green solvents only		Green solvents + antioxidants				Green solvents + pH (2–6)					
	A		B				C					
Time (h)	AE	AW	BEQ	BEP	BWQ	BWP	CE2	CE4	CE6	CW2	CW4	CW6
0	208.55	95.10	211.97	230.20	194.32	91.52	439.03	229.45	5.80	584.11	123.35	2.92
2	208.44	94.06	211.52	228.49	193.29	85.27	405.04	174.56	4.53	529.80	118.71	2.64
4	207.66	91.99	210.97	228.17	191.33	84.38	395.29	130.74	3.94	481.57	112.98	2.61
6	201.80	89.55	210.87	227.25	188.19	82.85	390.77	101.92	3.60	453.06	112.85	2.48
8	200.70	89.40	209.66	226.77	187.59	80.08	385.75	85.62	3.57	422.84	111.78	2.44
10	194.11	89.36	209.40	224.58	186.04	73.01	382.11	69.68	3.41	399.40	108.86	2.34
12	190.63	86.26	209.22	223.53	185.97	72.06	381.25	59.41	3.29	382.40	106.76	2.33
14	189.58	85.22	208.97	219.78	185.93	68.42	379.89	53.74	3.28	365.39	106.12	2.33
16	183.43	84.18	205.44	219.35	185.19	64.01	376.55	45.37	3.18	348.55	104.28	2.32
18	183.35	83.76	202.64	218.68	185.08	61.14	374.91	40.30	3.17	321.79	103.59	2.32
20	177.69	81.47	202.25	212.83	183.97	57.02	373.19	35.89	3.11	252.76	100.82	2.30
22	172.55	79.76	201.83	209.19	183.28	54.43	372.85	32.77	2.90	249.98	99.45	2.30
24	171.36	53.40	191.68	203.02	180.95	50.90	368.60	31.57	2.87	247.42	98.10	2.29
70	54.56	43.56	59.32	36.08	127.56	10.38	319.48	4.12	2.62	188.85	64.98	2.28
94	2.93	1.79	2.70	12.34	9.19	4.75	19.30	1.04	0.25	27.53	3.16	0.12
Descriptive statistics												
Mean	*169.82*	*76.59*	*183.23*	*194.68*	*171.19*	*62.68*	*357.58*	*73.08*	*3.30*	*350.36*	*98.38*	*2.27*
Sum	*2547.32*	*1148.87*	*2748.45*	*2920.25*	*2567.88*	*940.24*	*5363.71*	*1096.19*	*49.52*	*5255.44*	*1475.76*	*34.02*
SD	*59.39*	*25.23*	*62.95*	*69.78*	*47.50*	*25.47*	*96.81*	*63.33*	*1.15*	*142.64*	*29.44*	*0.62*
Minimum	*2.93*	*1.79*	*2.70*	*12.34*	*9.19*	*4.75*	*19.30*	*1.04*	*0.25*	*27.53*	*3.16*	*0.12*
Maximum	*208.55*	*95.10*	*211.97*	*230.20*	*194.32*	*91.52*	*439.03*	*229.45*	*5.80*	*584.11*	*123.35*	*2.92*

in the medium, presence of metals, and storage conditions [5,7,10,11]. Albeit different approaches have been utilized to enhance the stability of catechins, the factors affecting the catechins stability were studied merely in post-extraction processes. Herein, this study employs a novel approach where the factors affecting catechins extraction yield and stability are evaluated during a real-time/active extraction process. Three different models [A–C] were proposed and applied during the active extraction procedure for catechins from green tea leaves. The aim was to compare the extraction yield along with the stability and degradation of catechins in the three models. A novel USE technique was applied to extract catechins from green tea leaves. Ultrasonic adds the advantage of high energy ultrasonic waves (20-kHz) where the sample, time, and solvent used for extraction are reduced to the minimum while yielding more extract and a range of phytochemical extraction from the samples [9]. Prior to any extraction, the mediums were pre-prepared for all the models A-C followed by USE. For model-A; only green solvents (EtOH and H$_2$O) were used for extraction (without the addition of any antioxidants or pH changes), for model-B; the natural antioxidants (PPN and QT) were added into the green solvents whereas, for model-C; green solvents with different pH (2, 4, and 6) were prepared. Each sample from the three models weighed 20 mg which was dissolved in 40 mL of the respective solvents and processed with USE. In order to analyze the USE samples, UHPLC-MS/MS was used to develop and validate a green, sensitive, and accurate method for catechins quantification, followed by
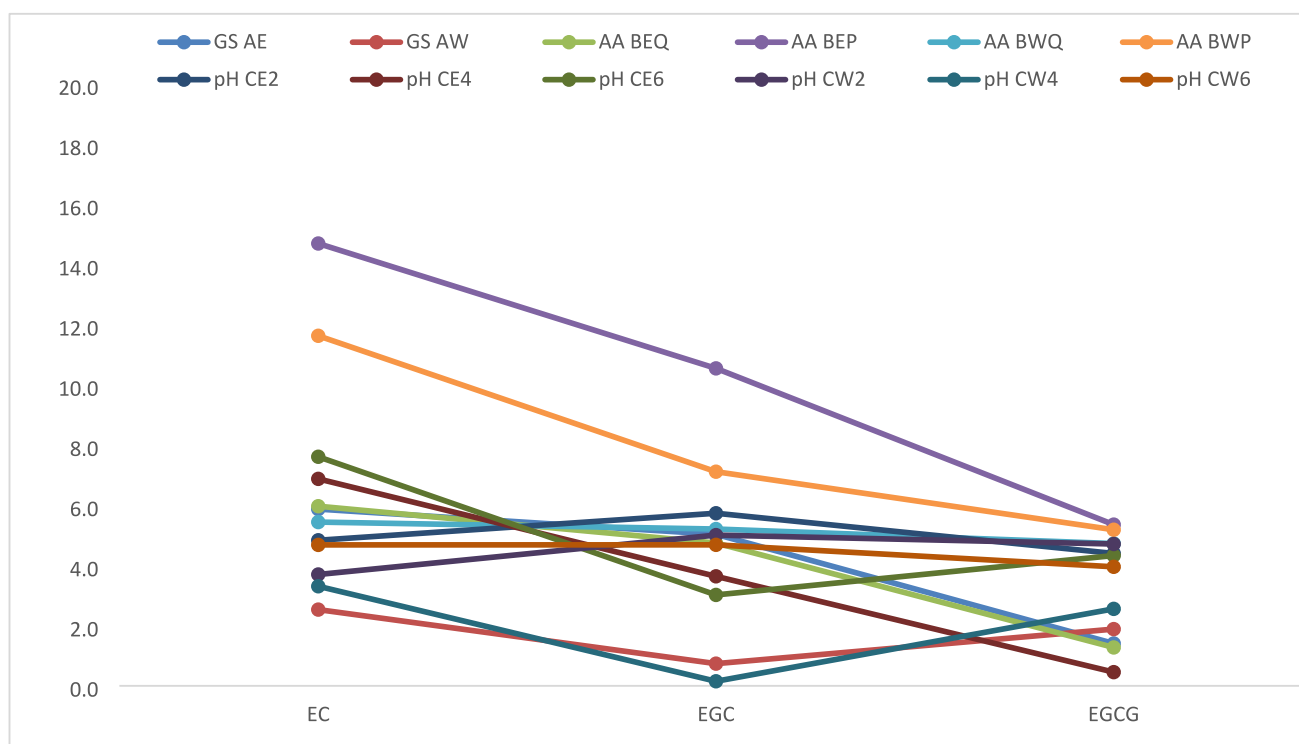
Fig. 2. Stability profile for catechins in model A-C; GS (green solvent), AA (antioxidant with green solvents), pH (different pH for green solvents used).

Table 6
Degradation profile (%) for catechins (EC, ECG, and EGCG) at time "24, 70, and 90 h".

| Model		Green solvents only		Green solvents + antioxidants				Green solvents + different pH mediums					
Catechins	Time	AE	AW	BEQ	BEP	BWQ	BWP	CE2	CE4	CE6	CW2	CW4	CW6
EC	24	8.18	16.52	16.02	17.41	10.04	8.43	15.46	49.81	63.71	63.38	7.56	13.25
	70	27.2	18.4	21.4	19.7	21.1	24.8	24.2	64.9	65.4	73.7	21.7	28
	94	94.1	97.5	94	85.3	94.6	88.4	95.2	93.1	92.4	96.3	96.7	95.3
ECG	24	10.35	6.38	11.02	19.85	6.52	16.09	11.99	54.47	95.05	59.58	8.57	19.89
	70	31	13.4	23.4	24.1	13.7	39.9	18.2	72.9	95.1	65.8	17.3	32.2
	94	95	99.3	95.3	89.4	94.8	92.9	94.3	96.4	97	95	99.8	95.3
EGCG	24	17.83	43.85	9.57	11.81	6.88	44.38	16.04	86.24	50.52	57.64	20.47	21.60
	70	73.8	54.2	72	84.3	34.4	88.7	27.2	98.2	54.9	67.7	47.3	21.9
	94	98.6	98.1	98.7	94.64	95.3	94.8	95.6	99.5	95.7	95.3	97.4	96

its stability and degradation profiling. The accuracy and precision, along with LODs, LOQs, and CC data suggested the developed method to be a reliable tool for catechins quantification. Any samples extracted in models A-C were filtered immediately without any treatment or processing, and subjected to the in-house developed analytical method. The first reading following USE was termed "0 h" followed by the analysis of the same sample at pre-determined time points of 0–94 h (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 70, and 94 h). The data generated were used to calculate the comparative catechins yield, stability, and % degradation in the three models.

In terms of catechins yield, model-A exhibited more amount for catechins in EtOH (AE) with the comparatively highest yield observed for EGCG. The order for solvent and catechins yields in model-A was; AE > AW and, EGCG > ECG > EC, respectively. Model-B revealed more yield for all the catechins in BEP (EtOH added with natural antioxidant PPN). Herein too, EGCG was observed the most abundant among catechins. The order for solvent and individual catechins with more yield was; BEP > BEQ > BWQ > BWP and, EGCG > ECG > EC, respectively. For model-C, both the green solvents produced more yield for catechins at a lower pH-2. An increase in the pH of the medium resulted in a decreased yield for catechins. Though it was pH-2 with the highest yield for catechins in model-C, CW2 (H$_2$O at pH-2) was more effective

followed by CE2 (EtOH at pH-2) for catechins yield. The data for individual catechins constructed a descending order of yield as; EGCG > ECG > EC in CW2, thereby showing a more abundance occurrence for EGCG, which is in-line with previous reports [12,13]. With regard to the yield in green solvents used, it's obvious that catechins may favor EtOH to be the solvent of choice for extraction; hence, more yield in this study was observed for EtOH as an extraction medium in all the three models. This data is in line with the previous literature reporting the order for catechins solubility to be EtOH > water > *n*-hexane [14]. Though the catechins yield was more for EtOH in model A and B, it was water at pH-2 showing the highest catechins yield throughout the models A-C followed by EtOH at pH-2. This indicates the profound effect of pH where a decrease in pH results in more yield for catechins. The lower pH of the medium imparts rigidity to the catechins structure and helps maintain its molecular structure [15], resulting in more stability and yield for catechins. Additionally, more stability for catechins has been reported in aqueous mediums when the pH was at lower side such as below 4 [16]. The lower pH of the aqueous media (pH-2) used in this study may be suggested to enhance catechins stability and maintain the more yield, as seen with the highest yield for CW2 in all the models of EC, ECG, and EGCG. The general yield for catechins was; model-C (pH) > model-B (AA) > model-A (green solvents).

EC
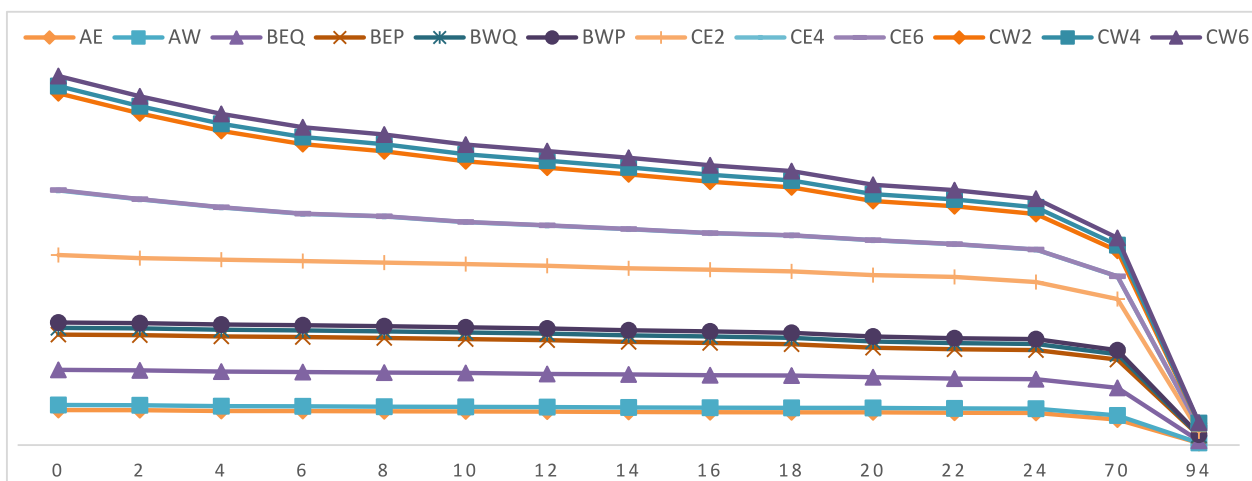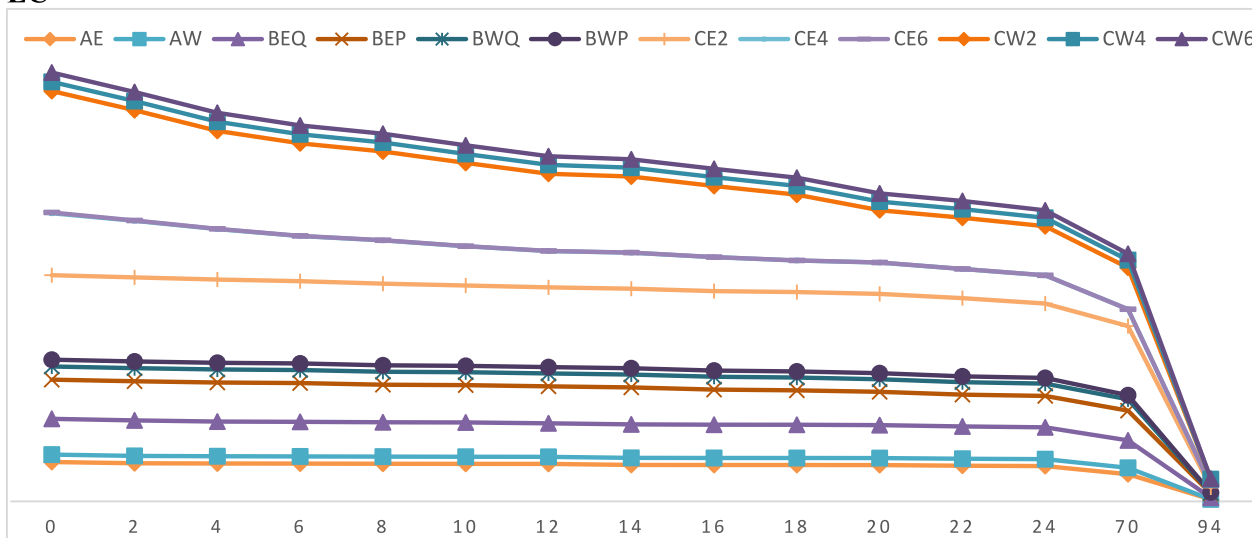
ECG
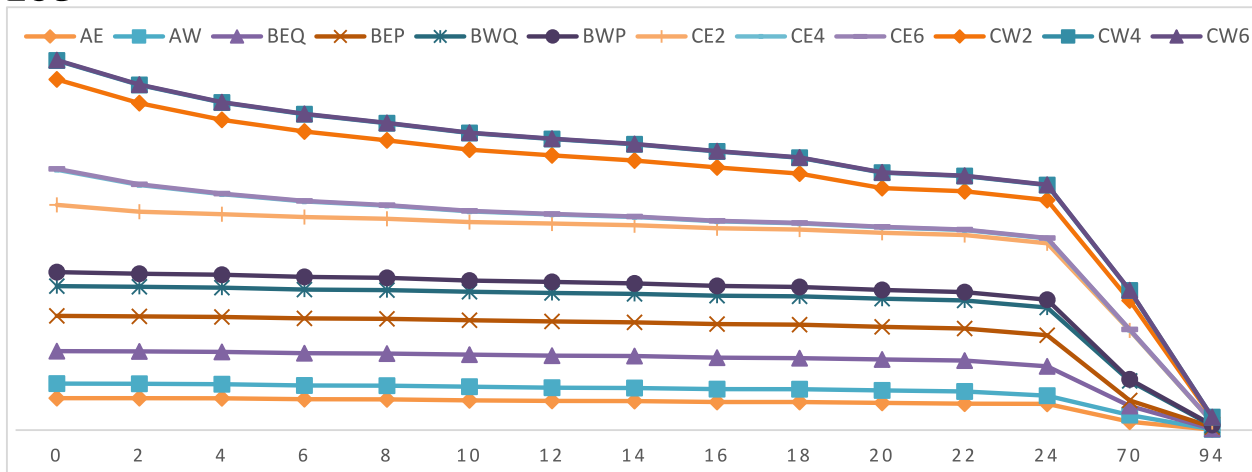
EGCG

Fig. 3. Yield with degradation profile at different time points for EC, ECG, and EGCG.

In terms of catechins stability, %recoveries were calculated where model-A was seen with a maximum recovery of 5.87 %, model-B with 14.70 %, and model-C with 7.61 %. The recoveries in these models were maximal for EC and ECG. For EGCG, there was a minimal recovery in model-A with a maximal recovery in model-B followed by model-C (model-B > model-C > model-A). Model-B represents the use of PPN

and QT as natural antioxidants during the active extraction process of catechins from green tea leaves. PPN was the most effective in terms of stability and %recoveries for catechins, EGCG in particular. The catechins extraction and its stability is influenced by a number of environmental factors during the extraction or processing of catechins sample. For instance, the presence of metals or free radicals in a medium may

Table 7
One-way ANOVA with Post-hoc Tukey's test for the three models of catechins extraction and stability ($P = 0.05$).

EC Model	Subset for alpha = 0.05			ECG Model	Subset for alpha = 0.05			EGCG Model	Subset for alpha = 0.05			
	1	2	3		1	2	3		1	2	3	4
9	0.36			**9**	0.44			**12**	2.40			
2	7.03			**6**	15.07			**9**	3.44			
6	7.72			**2**	17.34			**6**	62.54	62.54		
5	9.82			**12**	20.48			**8**		73.08		
11	10.56			**11**	21.41			**2**		76.94		
12	14.44			**5**	31.15			**11**		98.38		
3		49.74		**3**		81.56		**1**			169.82	
4		50.06		**4**		87.85		**5**			171.20	
1		50.27		**1**		89.00		**3**			183.22	
8		64.08		**8**		94.32		**4**			194.01	
10			87.713	**10**			188.64	**10**				350.36
7			94.633	**7**			194.87	**7**				357.57

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
EC	Between groups	182707.44	11	16609.76	61.06	**0.00**
	Within groups	45693.10	168	271.98		
	Total	*228400.55*	*179*			
ECG	Between groups	720325.10	11	65484.10	66.53	**0.00**
	Within groups	165358.27	168	984.27		
	Total	*885683.37*	*179*			
EGCG	Between groups	2275159.32	11	206832.66	48.92	**0.00**
	Within groups	710255.08	168	4227.70		
	Total	*2985414.41*	*179*			

Table 8
One-way ANOVA for catechins degradation in model A-C ($P = 0.05$).

		df	Mean Square	F	Sig
AE	Between Groups	2	5363.265	22.937	0.002
	Within Groups	6	233.822		
AW	Between Groups	2	5332.816	18.373	0.003
	Within Groups	6	290.246		
BEQ	Between Groups	2	5496.601	19.657	0.002
	Within Groups	6	279.628		
BEP	Between Groups	2	4151.973	9.288	0.015
	Within Groups	6	447.048		
BWQ	Between Groups	2	6484.022	171.632	0.000
	Within Groups	6	37.779		
BWP	Between Groups	2	3616.635	7.319	0.025
	Within Groups	6	494.162		
CE2	Between Groups	2	5861.388	662.490	0.000
	Within Groups	6	8.848		
CE4	Between Groups	2	810.186	3.447	0.101
	Within Groups	6	235.056		
CE6	Between Groups	2	589.692	1.837	0.239
	Within Groups	6	320.980		
CW2	Between Groups	2	1013.738	117.339	0.000
	Within Groups	6	8.639		
CW4	Between Groups	2	6213.585	58.721	0.000
	Within Groups	6	105.816		
CW6	Between Groups	2	5356.533	346.931	0.000
	Within Groups	6	15.440		

favor catechins oxidation with a drastic loss of catechins, especially in the absence of antioxidants in the medium. A previous study reported epimerization of EGCG to GCG with 5 % (6 h) and 90 % degradation for EGCG in the presence and absence of O_2, respectively [5]. Herein, the addition of PPN to the medium enhanced the catechins stability where EC and ECG were seen to be stable with a minor degradation till 70 h whereas, EGCG remained stable for 24 h with a degradation of 11.81 %. The enhancement in catechins stability for model-B may be due to the free radicals scavenging activity of PPN, a well-known antioxidant with a protective role against oxidative damage through free radical scavenging activity [17]. The free radical scavenging mechanism is supported by the additive effects of sugar and natural polyphenols (decreased radicals and chelation of metal ions in the medium) upon catechins and pecan oil stability [18,19]. Additionally, PPN is well known for its bioavailability enhancement property via inhibition of different metabolizing enzymes and P-glycoprotein inhibition [17], thereby adding an additional advantage for increasing the bioavailability of catechins when used concomitantly. The stability for catechins was observed to be EC > ECG > EGCG whereas, the stability potential for the models was model-B > model-C > model-A.

The degradation profile for catechins was evaluated with respect to loss on storage (%) at predetermined time points 0–94 h. The degradation for these catechins is shown in Table 6. Three time points (24, 70, and 94 h) were selected due to the stability of catechins till 24 h throughout the three models except CE4, CE6, CW2, and CW4 where a degradation of >50 % was observed. Likewise, EC and ECG exhibited a stable behavior till 70 h, following which a significant degradation was observed; however, the degradation rate for EGCG initiated at the end of 24 h where most of the EGCG samples revealed a degradation of >50 % with an incremental increase (>70 %) at 70 h. EGCG remained the more unstable among the catechins with more %degradation, however, the use of antioxidants during the active extraction process may be a potential strategy to decrease the degradation rate for EGCG. Yet again, this study enabled EGCG to remain stable for 24 h in BWQ (6.88 % loss) and BEP (11.81 % loss), which is a more significant reduction in degradation as compared to previous reports [5]. Apart from EGCG, the EC and ECG were also noted to have much lower degradation as compared to model-C and A. The presence of free radicals or low O_2 amount in a medium is the most widely reported cause for instability of catechins, EGCG in particular hence, a number of antioxidants has been employed with the aim to scavenge the free radicals in the medium and enhance EGCG stability. The use of sugars, natural polyphenols, and antioxidant enzymes (superoxide dismutase) for EGCG has been reported with considerable stability for EGCG in the medium [5,18,19]. The application of natural antioxidants PPN and QT played the role of free radical scavengers in the catechins medium and imparted more stability to catechins and EGCG therein. As this study utilized one concentration for the natural antioxidants PPN and QT, the authors do

believe that the use of more concertation (high % of antioxidant in the medium) may further improve the stability and prevent degradation for catechins.

The statistical models of descriptive analysis and one-way ANOVA exhibited significant differences ($P < 0.05$) for the catechins yields, stability, and degradation profile. It was model C (CE2 and CW2) with a significant difference in terms of catechins yield, whereas, for stability of catechins model-B (BEP, BWP, and BWQ) exhibited a significant difference within the groups. In a wider context, more yield for catechins was observed in model-C (CE2 and CW2) i.e. at a lower pH; however, it was model-B (PPN and QT) imparting more stability (%recovery) and lower degradation for catechins, especially BEP, BWP, and BWQ. The study demonstrates the effective use of natural antioxidants for enhancing the yield and stability of catechins.

5. Conclusion

The effect of green solvents, pH of the solvents used, and natural antioxidants was evaluated during the in-process extraction of catechins from green tea leaves. Water as a green solvent at lower pH-2 produced the highest yield for catechins where EGCG was seen with the highest yield among the three catechins. It is noteworthy to mention that the aqueous medium at low pH revealed a high yield, and the stability for catechins was high in the presence of natural antioxidants. PPN was more potent in preventing catechins degradation, more importantly for EGCG which is known for its unstable behavior among catechins group. The degradation as well as stability for catechins may be improved further, following an extensive study for the combined effect of different concentrations of these natural antioxidants in aqueous mediums with different pH values.

CRediT authorship contribution statement

RA (conceptualization); MA and AA (literature review, introduction and discussion write up); EA and FA (data curation for USE and samples preparation); AM, AMA, HS (data curation and formal analysis for LCMSMS MDMV and USE samples analysis); RA (statistical analysis, M&M, results, and discussion write up); MA and AA (review, editing, and approval of the final manuscript).

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Availability of data

The datasets used to generate any conclusions are provided in the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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