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Improved quantification of catechin and epicatechin in red rice (*Oryza sativa* L.) using stable isotope dilution liquid chromatography-mass spectrometry

Tae Jin Kim[1,2†], Ye Jin Kim[1†], Woo Duck Seo[3], Sang Un Park[4*] and Jae Kwang Kim[1*]

Abstract

Epimerization can change the catechin content and composition of samples during extraction and analytical analyses. To control the effect of epimerization, we developed a novel and stable isotope dilution liquid chromatography-mass spectrometry (LC–MS) method using catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ as stable-isotope-labeled internal standards (SIL-ISs). When the SIL-ISs were used, the catechin and epicatechin contents were stable (104–109% and 100–109% of the initial concentration, respectively) despite long storage times. In contrast, when L-2-chlorophenylalanine was used as an internal standard, catechin and epicatechin concentrations of 88–97% and 164–277% of the initial concentration, respectively, were obtained after long storage times. Furthermore, the least significant epimerization effect and highest extractability were observed when extraction was performed at 70 ℃ for 30 min. The recoveries for red rice using the developed isotope dilution LC–MS method at two different concentrations were between 100.72 and 118.67%, with relative standard deviations less than 3.67%.

Keywords: Catechin, Epicatechin, Epimerization, Stable isotope, Liquid chromatography-mass spectrometry

Introduction

Catechins are flavanols, a class of flavonoids. (+)-Catechin and (−)-epicatechin are known as major catechin compounds [1]. Catechins have strong antioxidant activity due to their one-electron reduction potential. Hence, catechins are reported to confer various health benefits [2–7]. In addition, catechins are considered inexpensive, readily applicable, and safe phytochemicals. Various analytical methods have been developed to determine the catechin contents in plants and food products [8–19].

Catechins epimerization is an important factor influencing the composition of catechins in plants. Epimerization can change non-epistructured catechins to epistructured catechins, and vice versa. It is reported to occur over a pH range of 5.4–11.0 and between 34 and 100 ℃ [20, 21]. This changes the catechin content and overall composition of the samples during extraction and analysis. Therefore, several methods for controlling the epimerization of catechins during extraction and analysis have been reported [1, 22].

The analytical techniques for the qualitative-quantitative analyses of catechins have been reported using nuclear magnetic resonance (NMR) spectroscopy (^{1}H- and ^{13}C-), high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC–MS) [8–15]. NMR spectroscopy has been

†Tae Jin Kim and Ye Jin Kim are contributed equally to this work

*Correspondence: supark@cnu.ac.kr; kjkpj@inu.ac.kr

[1] Division of Life Sciences and Convergence Research Center for Insect Vectors, College of Life Sciences and Bioengineering, Incheon National University, Incheon 22012, Republic of Korea
[4] Department of Crop Science and Department of Smart Agriculture Systems, Chungnam National University, 99 Daehak-Ro, Daejeon, Yuseong-Gu 34134, Republic of Korea
Full list of author information is available at the end of the article

commonly used to confirm chemical structures for epimerization. LC–MS provides accurate mass, isotopic distribution, additive ion information, and full scan data. Furthermore, LC–MS has been commonly used with stable isotope dilution techniques [23]. This technique is an accurate method for determining the concentration of a given analyte in any type of matrix. It is based on the direct proportionality of the mass fraction and signal intensity ratio of the natural target analyte and an isotopically labeled form of the target analyte.

An analytical method using a stable isotope-labeled internal standard (SIL-IS) can be a useful tool for the precision and accuracy detection of a target analyte. A SIL-IS is a derivative of a target analyte in which several atoms have been replaced with stable isotopes. For this reason, the SIL-IS and target analyte have nearly the same chemical structure and properties and are expected to be similarly affected by epimerization. As a result, overlapping peaks (SIL-IS and target analyte) cannot identify in a highly complex mixtures and the desired components cannot be accurately quantified. The HPLC techniques with UV or fluorescence detectors and NMR spectroscopy only measure integrated overall signal of individual compounds [24]. On the other hand, LC–MS can identify individual metabolite in complex matrix due to accurate measurement of molecular weight. LC–MS has high selectivity and sensitivity. In addition, MS is inherently much more selective and sensitive than NMR spectroscopy [25]. However, stable isotope dilution methods to overcome catechins epimerization issues have yet to be reported.In this study, we developed a novel method for determining catechin and epicatechin contents using LC–MS with SIL-IS to overcome the epimerization effect. Moreover, we evaluated the stability and contents of catechin and epicatechin in red rice extracts over different storage times to investigate the effect of storage time on the epimerization of catechins.

Materials and methods

Catechin and epicatechin (≥ 98%) were purchased from ChemFaces (Wuhan, China). In addition, the materials used in this study include L-2-chlorophenylalanine (Sigma-Aldrich, St. Louis, MO, USA), catechin-2,3,4-$^{13}C_3$ (Cambridge Isotope Laboratories, Inc, MA, USA), epicatechin-2,3,4-$^{13}C_3$ (Cambridge Isotope Laboratories, Inc., MA, USA), water (Honeywell Burdick and Jackson, NJ, USA), and red rice (Cultivar: Jagwangdo, obtained from the Agricultural Genetics Resources Center at the National Academy of Agricultural Science, Suwon, Korea). The extraction of catechin and epicatechin was performed according to a previously reported method with slight modifications [26]. Briefly, the rice powder sample (0.01 g) was extracted with 1.0 mL of water containing 1.2 M HCl. In addition, L-2-chlorophenylalanine and the SIL-ISs, catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$, were added to 1.00 μg/mL. To investigate the ideal extraction conditions to achieve the least epimerization effect and highest extraction efficiency, we tested different extraction temperatures and extraction times. The samples were vortexed and incubated at 30, 50, 70, and 100 ℃, with shaking at 1200 rpm, for 10, 20, 30, 60, and 120 min. Subsequently, the samples were centrifuged for 10 min at 16,000 × *g* and 4 ℃. The upper layer was filtered through a 0.45 μm syringe filter and transferred into an autosampler vial for LC–MS analysis. Catechin and epicatechin were separated on a Develosil ODS-UG-5 column (2.0 × 250 mm, Nomura Chemical, Seto, Japan) by an Agilent 1260 Infinity HPLC System (degasser, quaternary pump, and autosampler) equipped with an Agilent 6120 single quadrupole MS with electrospray ionization (Fig. 1). To investigate the effects of storage time on epimerization, the catechin extract was placed in an autosampler tray for 0, 4, 8, and 12 h before injection. To validate the method, six calibration points of different concentrations of catechin and epicatechin (0.16–5.00 μg/mL) and a fixed volume internal standard (1.00 μg/mL) were prepared. Method validation was performed following Appendix F of the Association of the Official Analytical Collaboration (AOAC) guidelines [27]. Full experimental details are provided in the Additional file 1.

Results and discussion

Effect of extraction temperature and time

The catechin and epicatechin contents of red rice obtained from various extraction temperatures and times are shown in Fig. 2. We found that the catechin content gradually increased according to the extraction time at 30 ℃ (Fig. 2A). However, epicatechin was not detected at 30 ℃. At 50 ℃, more catechin and epicatechin were extracted than at 30 ℃ under identical extraction times (Fig. 2B). After 120 min, the extract processed at 50 ℃ contained more than double the catechin content extracted at 30 ℃. The concentration of catechin at 70 ℃ increased up to 30 min and then rapidly decreased up to 120 min (Fig. 2C). In contrast, the epicatechin concentration at 70 ℃ increased up to 60 min and then remained constant up to 120 min. These results indicate that catechin was epimerized to epicatechin at high temperatures. Wang et al. reported that the level of catechins decreased, whereas that of its isomer increased as a result of increasing temperature [20]. Therefore, after 30 min, the epicatechin content was increasing due to epimerization rather than decreasing due to degradation at high temperatures as catechin did. In a previous study, continued heating after 30 min degraded epicatechin and

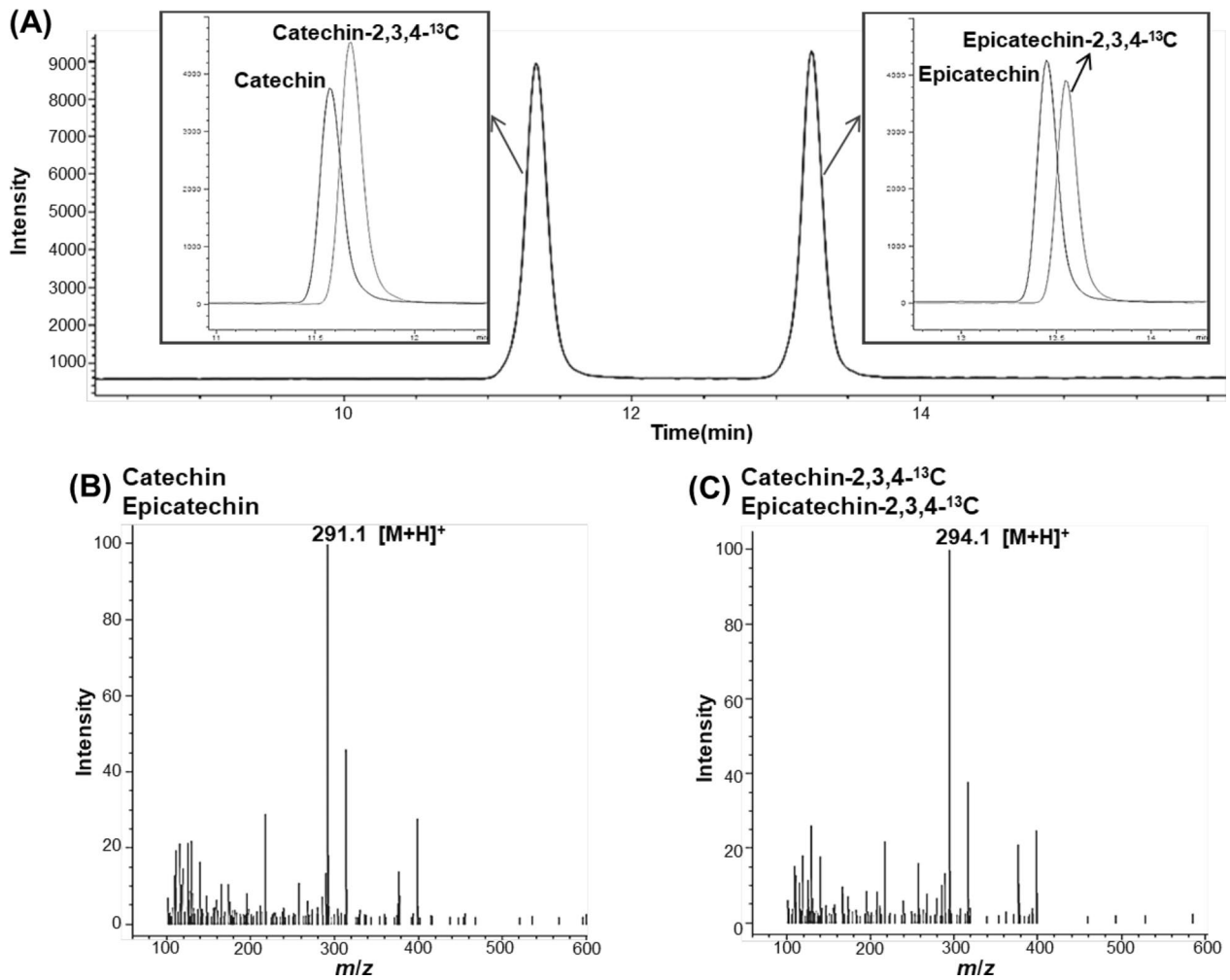

Fig. 1 Chromatograms and mass spectra of catechin, epicatechin, and SIL-IS (catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$). **A** Selected ion chromatograms of catechin, epicatechin (*m/z* 291.1) and SIL-IS (*m/z* 294.1). Mass spectra of **B** catechin, epicatechin, and **C** SIL-IS. *SIL-IS* stable-isotope-labeled internal standard

formed 3,4-dihydroxy benzaldehyde and protocatechuic acid [28]. In this case, the increase in epicatechin content was not proportional to the decrease in catechin content due to epimerization. Finally, when the extraction temperature was 100 ℃, all the catechins degraded (Fig. 2D). In previous studies, the monomeric forms of flavonoids (catechin and epicatechin) were less stable than the polymeric forms when exposed to light, heat, and basic conditions [29]. In another study, the degradation and epimerization of catechin significantly increased after 30 min at elevated temperatures (80 ℃ and 100 ℃) [28]. In summary, poor extraction efficiency was observed for catechin and epicatechin at 30 and 50 ℃. The highest extraction efficiency was obtained at 70 ℃ and 30 min. Furthermore, at 100 ℃, the extract degraded due to the elevated temperature. Therefore, the ideal extraction conditions for reduced epimerization were 70 ℃ and 30 min.

Epimerization of catechins after extraction

To investigate the effect of storage time on the extraction of catechins from red rice, the aqueous catechin extracts were placed in an autosampler tray for 0, 4, 8, and 12 h before injection (Fig. 2E and F). The changes in the catechin and epicatechin contents were expressed as the percentage remaining (%). Figure 2 E shows the change in catechin content, quantified by SIL-IS and L-2-chlorophenylalanine, according to storage time. The initial catechin concentration (104–109%) was maintained for all storage times tested. However, the catechin content quantified by L-2-chlorophenylalanine was decreased by 12% after 12 h of storage. The changes in epicatechin

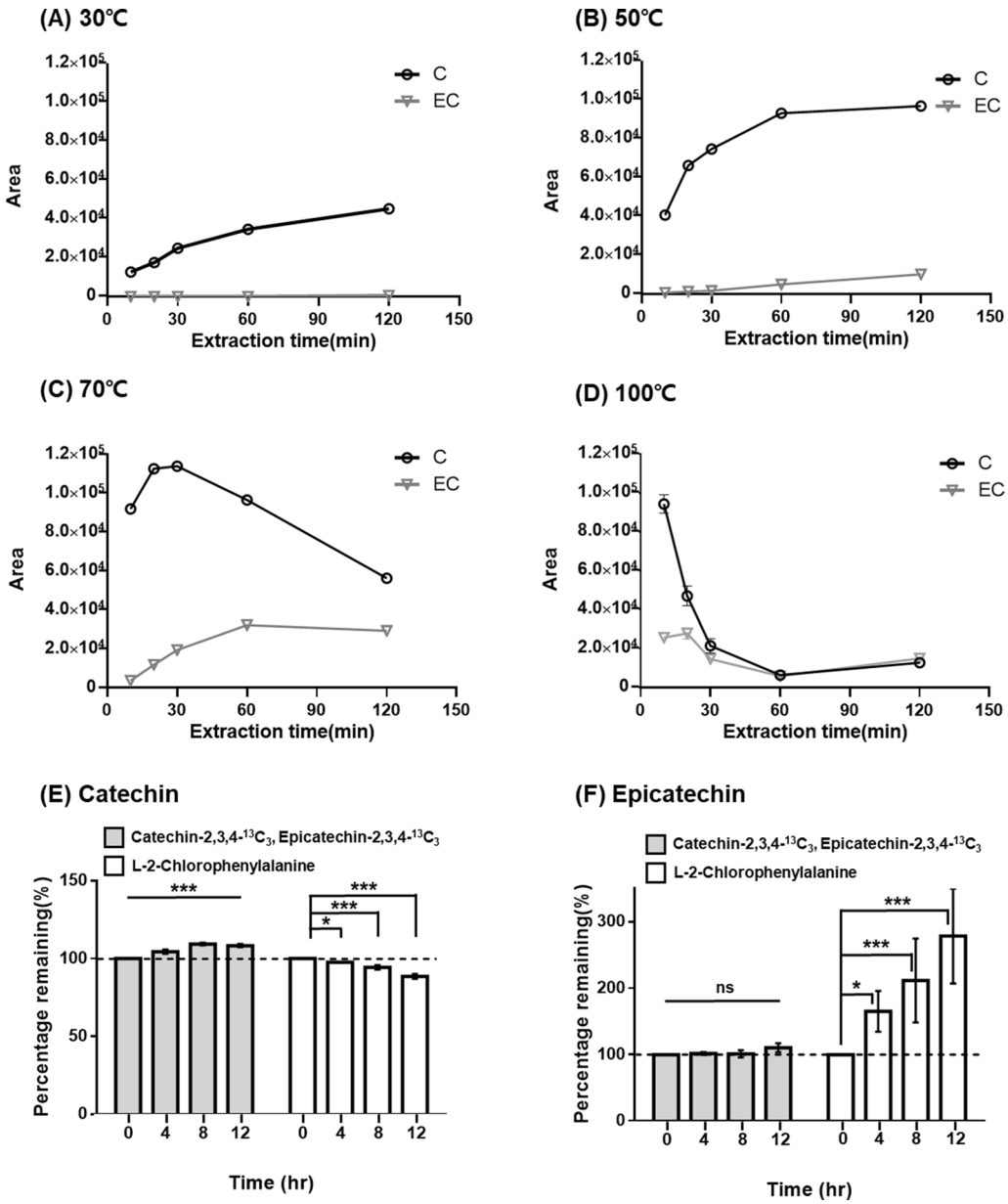

Fig. 2 Effect of different extraction temperatures and times on the extraction efficiency of catechin and epicatechin. Area of catechin, epicatechin, catechin-2,3,4-$^{13}C_3$, and epicatechin-2,3,4-$^{13}C_3$ at 30 ℃ **A**, 50 ℃ **B**, 70 ℃ **C**, and 100 ℃ **D** as extraction time progressed. Catechin (E) and epicatechin (F) stability using L-2-chlorophenylalanine and SIL-IS (catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$) as an internal standard. 100% represents the concentration at 0 h. An increase above 100 means that the catechin and epicatechin content has increased. Percentages under 100 indicate that the catechin and epicatechin content has decreased. Statistical significance was determined by two-way ANOVA followed by Bonferroni post-tests to compare each time point to 0 h (*$p < 0.05$, ***$p < 0.001$). All data are expressed as mean value of three experiments ± SD. *C* catechin, *E* epicatechin, *SIL-IS* stable-isotope-labeled internal standard

content quantified by SIL-IS and L-2-chlorophenylalanine, are shown in Fig. 2F. When SIL-IS was employed, the percentage remaining of epicatechin (100–109%) was stable over time. In contrast, the epicatechin quantified by L-2-chlorophenylalanine was significantly increased by approximately three-fold (164–277%) during the storage period. These results indicate that the catechin in red rice was epimerized to epicatechin during storage. Furthermore, the use of L-2-chlorophenylalanine, as an internal standard for accurate quantification, did not adequately compensate for the controlled epimerization of catechin to epicatechin. In contrast, the use of SIL-ISs led

to the consistent catechin and epicatechin contents of the samples stored over various times. These results revealed that the use of SIL-ISs compensated for the epimerization effect.

Method validation

LC–MS was performed with catechin and epicatechin standards (0.02–5.00 μg/mL). Additionally, catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ as SIL-ISs (1.00 μg/mL) were used for the calibration curve. The following regression equations were obtained using catechin-2,3,4-$^{13}C_3$: $y = 0.7598x + 0.014$ and epicatechin-2,3,4-13C_3: $y = 0.9389x + 0.0317$. Using SIL-ISs, the r^2 values of 0.9999 and 0.9997 were obtained for catechin and epicatechin, respectively. The limit of detection and limit of quantification were 0.034 and 0.112 μg/mL, respectively, for catechin, and 0.024 and 0.073 μg/mL, respectively, for epicatechin. To determine the precision (RSD %) and accuracy (recovery %), we assessed four different concentrations (0.63, 1.25, 2.50, and 5.00 μg/mL) of catechin and epicatechin within the calibration curve (Table 1). The precision of the catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ standards (≤ 3.17%) was within the acceptable range according to the AOAC guidelines (for 1 μg/mL: ≤ 11.0%, for 10 μg/mL: ≤ 7.3%). In addition, the accuracy values for stable isotope dilution LC–MS (catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$: 99.4–102.24%) were also within the acceptable ranges (for 1 μg/mL: 80–110%).

Table 1 LC–MS method precision (RSD, %) and accuracy (recovery, %) for catechin and epicatechin using SIL-IS

Concentration (μg/mL)	Precision (RSD, %)		Accuracy (Recovery, %)	
	Catechin[a]	Epicatechin[b]	Catechin[a]	Epicatechin[b]
5	1.53	0.53	99.67 ± 1.52	99.40 ± 0.52
2.5	2.17	1.30	101.48 ± 2.20	102.24 ± 1.33
1.25	1.29	1.71	99.82 ± 1.29	101.11 ± 1.73
0.63	2.62	3.17	99.83 ± 2.61	100.43 ± 3.18

[a] Catechin-2,3,4-$^{13}C_3$

[b] epicatechin-2,3,4-$^{13}C_3$

SIL-IS stable-isotope-labeled internal standard

To evaluate the precision and accuracy of the intra- (n = 3) and inter-day (n = 5) measurements, the red rice sample was spiked with two different concentrations (0.1 and 0.2 μg/mL) of catechin and epicatechin (Table 2). The precision values of the catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ standards were ≤ 6.66% in the intra- and inter-day (AOAC guidelines for 1 μg/mL: ≤ 11.0%). In addition, the accuracy values for the catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ assays in the intra- and inter-day measurements were good.

In this study, we developed a novel and stable isotope dilution LC–MS method using catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$ as SIL-ISs. The SIL-ISs compensated for the epimerization effect of the catechins because they had nearly the same chemical properties and underwent epimerization in a similar manner. To control the epimerization of catechin and epicatechin, we investigated the stability of catechin and epicatechin over 12 h and the ideal extraction conditions to reduce epimerization. The results indicate that the use of SIL-IS enabled the measurement of stable catechin and epicatechin contents over 12 h, unlike when L-2-chlorophenylalanine was used as an internal standard. In addition, the ideal extraction conditions to reduce epimerization were 70 ℃ and 30 min. The developed novel and stable isotope dilution LC–MS method was validated according to the AOAC guidelines and exhibited acceptable validation results. In future work, experiments need to be devised

Table 2 Precision (RSD, %) and accuracy (recovery, %) for catechin and epicatechin in red rice using SIL-IS[c]

	Volume added	Intra-day[a]		Inter-day[b]	
		Precision	Accuracy	Precision	Accuracy
	(μg/mL)	(RSD, %)	(Recovery, %)	(RSD, %)	(Recovery, %)
Catechin	0	0.48	–	0.77	–
	0.1	0.94	100.80 ± 0.95	0.68	100.72 ± 0.79
	0.2	0.64	102.10 ± 0.66	0.58	101.97 ± 0.83
Epicatechin	0	6.66	–	5.30	–
	0.1	3.67	117.11 ± 4.29	3.87	118.67 ± 6.26
	0.2	2.07	114.71 ± 2.38	3.19	116.00 ± 3.25

[a] Within batch (n = 3)

[b] between batches (n = 5)

[c] catechin-2,3,4-$^{13}C_3$ and epicatechin-2,3,4-$^{13}C_3$

SIL-IS stable-isotope-labeled internal standard

to explain the chemical patterns of catechin formation by epimerization and degradation.

Abbreviations
AOAC: Association of the Official Analytical Collaboration; LC–MS: Liquid chromatography-mass spectrometry; SIL-IS: Stable-isotope-labeled internal standard.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00754-2.

Additional file 1. Experimental details for the analysis of catechin and epicatechin.

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Author contributions
TJK carried out the experiments, prepared most of the data, and wrote the paper; YJK carried out the experiments, and prepared the data; JKK and SUP managed the research process, and reviewed and edited the paper. All authors read and approved the final manuscript.

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Availability of data and materials
The data and materials used in this study are available under permission from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that there is no competing interest.

Author details
[1]Division of Life Sciences and Convergence Research Center for Insect Vectors, College of Life Sciences and Bioengineering, Incheon National University, Incheon 22012, Republic of Korea. [2]Bio-Resource Industrialization Center, Nakdonggang National Institute of Biological Resources, Sangju-si, Gyeongbuk 37242, Republic of Korea. [3]Division of Crop Foundation, National Institute of Crop Science, Rural Development Administration, Wanju, Jeonbuk 55365, Republic of Korea. [4]Department of Crop Science and Department of Smart Agriculture Systems, Chungnam National University, 99 Daehak-Ro, Daejeon, Yuseong-Gu 34134, Republic of Korea.

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