KOREAN J. FOOD SCI. TECHNOL. Vol. 53, No. 6, pp. 682~687 (2021)
https://doi.org/10.9721/KJFST.2021.53.6.682

KOREAN JOURNAL OF
한국식품과학회지
FOOD SCIENCE AND TECHNOLOGY

Validation and measurement uncertainty of HPLC-UV method for quercetin quantification in various foods

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Abstract The purpose of this study was to validate a high-performance liquid chromatography (HPLC) method for the quantitative analysis of quercetin in various foods. The method was based on HPLC-UV (360 nm). The method was validated using candy, beverage, and sausage which were tested for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy, and the measurement uncertainty was assessed. Matrix-matched calibration was also applied. The calibration curves (0.5-50 mg/L) showed good linearity ($r^2 \geq 0.9998$). LOD and LOQ ranged from 0.15 to 0.31 mg/kg and from 0.44 to 0.93 mg/kg, respectively. The average accuracy and precision at 0.5, 2.5, and 10 mg/kg ranged from 84.3 to 102.0% and 0.7 to 3.0 relative standard deviation (RSD%), respectively. This study confirmed the applicability of the proposed method by applying it to commercial products, such as teas and beverages. Thus, the proposed analytical method is suitable for quantifying quercetin in various foods.

Keywords: quercetin, food additive, measurement uncertainty, validation

Introduction

Quercetin (3,3',4',5,7,-pentahydroxyflavone) is a natural polyphenolic flavonoid with a molecular formula of $C_{15}H_{10}O_7$ (Dai and Row, 2019). It is found in various foods, such as berries, capers, nuts, onions, and many flowers and leaves (Li et al., 2016). Quercetin is a potent antioxidant and displays anti-inflammatory, anticancer, antiviral, psychostimulant, cardioprotective, neuroprotective, and numerous other biological effects (Davis et al., 2009). To increase the health benefits of processed foods, quercetin has been added to cereal bars (Egert et al., 2012), steamed bread (Lin et al., 2018), and processed cheese (Přikryl et al., 2018), confirming its practicality and applicability as a functional food additive. Based on its history of use as a food additive (Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives [JECFA], 1977), quercetin has been designated "Generally Recognized as Safe (GRAS)" by the U.S. Food and Drug Administration (FDA) and excluded from the Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements (Lai and Wong, 2021).

For analytical techniques, high-performance liquid chromatography (HPLC), HPLC with ultraviolet (UV) (Kwak et al., 2017), diode array (Sharifuldin et al., 2016), and mass spectrometry detection (Aguirre-Hernandez et al., 2010), and gas chromatography-mass spectrometry (Tayade et al., 2013) were utilized to analyze quercetin in samples, such as onions and herbal medicinal plants. In the HPLC method described by Kwak et al. (2017), the binary mobile phase consisted of 5% formic acid and 100% methanol, and the analysis was performed under gradient conditions with a run time of 35 min. Additionally, Sharifuldin et al. (2016) reported a binary mobile phase composed of 0.3% formic acid and 100% acetonitrile and a run time of 20 min.

Quercetin is a lipophilic compound with low solubility in water (0.01 mg/mL, 25°C) (Gao et al., 2011), moderate solubility in ethanol (4.0 mg/mL, 37°C,) and high solubility in dimethyl sulfoxide (150 mg/mL, 25°C) (Ferry et al., 1996; Priprem et al., 2008). Several sample preparations, such as solid-phase extraction (Molinelli et al., 2002) and liquid-liquid extraction (de Souza Dias et al., 2012) for wines, microwave-assisted extraction (Du et al., 2009) for medicinal plants, and ultrasonication-assisted solvent extraction (Vasantha Rupasinghe et al., 2011) for apple peels have been used for the analysis of quercetin. Especially, ultrasonic-assisted extraction is an affordable, simple, and efficient alternative to conventional extraction techniques and has been widely used to extract various phenolic compounds from several parts of plants, such as leaves, stems, and fruits (Aybastıer et al., 2013). Additionally, to extract quercetin from dried *Raphanus sativus* leaves, a common cruciferous vegetable, methanol was superior to ethanol, water, and chloroform as the extraction solvent (Sharifi et al., 2016).

In addition to the various chromatographic techniques and extraction methods for quercetin analysis, a number of sample preparation techniques have been reported depending on the matrix, such as food, plasma, and pollen (Abdelkawy et al., 2017;

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Received September 15, 2021; revised September 27, 2021; accepted October 5, 2021

Chen et al., 2015; Tokuşoğlu et al., 2003; Wach et al., 2007).

Therefore, this study validated an analytical method for the quantification of quercetin in liquid, powder, and solid (fat-containing) food matrices. The specific aims of the study were to 1) develop a conventional HPLC-UV method to quantify quercetin in food, 2) validate the method using matrix-matched calibration, 3) evaluate the measurement uncertainty, and 4) apply the proposed and validated method to quantify the quercetin contents in various foods.

Materials and Methods

Materials and chemicals

Quercetin standard (Q4951, 99%; CAS No. 117-39-5) and formic acid (F0507, ≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and water were obtained as HPLC-grade solvents from JT Baker Chemical Co. (Radnor, PA, USA). To apply the proposed method, 22 food products (including nine leached teas, five onion skins, four liquid teas, and four processed products) distributed in Korea were purchased from a local market or online in 2021.

Sample preparation

Sample preparation was performed according to the published protocols (Glavač et al., 2017; Kwak et al., 2017; Sharifi et al., 2016) with the following modifications. About 2 g of the homogenized sample was added to 30 mL methanol, followed by ultrasonication-assisted extraction at 65°C for 10 min. After cooling at room temperature, the extract was adjusted to 40 mL, mixed, and filtered through a 0.45-µm membrane filter before HPLC analysis.

Analytical instruments

The HPLC analytical conditions were applied following Kwak et al. (2017) with some modifications. Samples were analyzed using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a pump, autosampler, column compartment, and UV detector. Analytes were separated on a Capcell Pak C18 column (Osaka Soda, Osaka, Japan, 250×4.6 mm, 5 µm) set at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B). The gradient program was 0-19 min, 80-40% A; 19-20 min, 40-0% A; 20-22 min, 0-40% A; 22-23 min, 40-80% A, followed by column equilibration with 80% A for 6 min. The flow rate and the injection volume were 0.8 mL/min and 10 µL, respectively. The wavelength was measured at 360 nm. Quercetin was identified based on retention time and UV-VIS spectra.

Validation

The proposed method was validated by a single-laboratory (in-house) usage, according to the International Conference on Harmonization (ICH) guideline Q2(R1) (ICH, 2005) and the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) guideline (AOAC, 2016). The specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision were determined.

Specificity is the ability to evaluate the target analyte unequivocally in terms of interfering components, which typically include impurities, the matrix, and degradation products. By comparing blanks, standard solutions, and samples, it was confirmed that the method was suitable for the specific analysis of quercetin. Linearity refers to a measurement that is proportional to the concentration of the analyte. The quercetin content was analyzed at six concentrations in the range of 0.5-50 mg/L, with seven replicates. LOD and LOQ were calculated by dividing the standard deviation (SD) of the y-intercept of the calibration curve by the average value of the slope after seven repeated analyses at the three lowest concentrations of the calibration curve prepared according to ICH Guideline Q2 (R1) (ICH, 2005). LOD and LOQ were multiplied by 3.3 and 10, respectively.

The matrix effect is defined by the European Commission (Sante, 2015) as a change that occurs when an interfering substance is present in the sample. It was calculated by comparing the slope of the calibration curve of the standard solution with the slope of the calibration curve obtained by adding different concentrations of the standard to the sample, and calculated as follows: (González-González et al., 2019)

$$\text{Matrix effect (\%)} = \left[\frac{\text{Slope}_{\text{standard}}}{\text{Slope}_{\text{spiked}}} - 1\right] \times 100$$

Accuracy is the degree to which the measured value is close to the standard value. The accuracy of the method was expressed as the recovery rate (%) of quercetin in beverage, candy, and sausage spiked with 0.5, 2.5, and 10 mg/kg of quercetin standard solution. Precision was evaluated as the intra-day and inter-day relative standard deviations (RSD%) of the recovery rate. For intra-day precision, six repetitions were performed for 1 day, and for inter-day precision, three repetitions were performed on 3 days. The Horwitz ratios, HorRat (r), were also used to calculate the repeatability. The HorRat is an index of the RSD per unit of the predicted RSD, given by $2C^{-0.15}$, where C is the mean concentration expressed as a mass fraction (AOAC, 2016).

Inter-laboratory validation

Inter-laboratory validation for accuracy and precision was assessed by comparing the analysis results of the same sample using the same analysis method in three different laboratories (Lab A, Lab B, and Lab C). A beverage was selected as the sample, and a recovery experiment was performed by adding 0.5, 2.5, and 10 mg/kg of quercetin standard. The recovery experiment was repeated three times. Then, the recovery rate and RSD% were obtained to confirm the accuracy and precision.

Measurement uncertainty assessment

To complete the validation of the method, the measurement uncertainty was estimated using mathematical processing and statistical methods based on the EURACHEM (A Focus for Analytical Chemistry in Europe) method (Ellison and Williams, 2012). The estimation of this parameter renders data from inter-laboratory studies comparable and leads to better measurement

reliability, and it is also a requirement of the ISO/IEC 17025:2017 standard. After determining the uncertainty of the standard stock solution preparation (*uSSS*), sample preparation (*uSP*), calibration curve (*uCal*), and repeated measurement of samples (*uRP*), the expanded uncertainty (*Uc*) was estimated and calculated using the coverage factor (k) of 2 at the 95% confidence level.

Results and Discussion

HPLC validation

The blank, standard solution (10 mg/kg), and samples are compared in Fig. 1. Specificity was validated because no substances interfered with the peak retention time of quercetin in each sample. The retention time of quercetin was about 21.1 min. The method was selective for the analysis of quercetin in various foods.

The linearity of each matrix added with quercetin was repeated seven times in total with six concentrations in the range of 0.5-50 mg/L, and the results are shown in Table 1. Regression coefficient (r^2) values ranged from 0.9998 to 1.0000. The linearity met the acceptance criterion of $r^2 \geq 0.995$ established by the FDA in the "Methods, Method Verification, and Validation" protocol (FDA, 2014).

LOD and LOQ were calculated using the SD of the intercept of the repeated calibration curve and the mean of the slope. As a result, values of 0.15 to 0.31 mg/kg and 0.44 to 0.93 mg/kg were obtained, respectively. These results were similar to previous research (Buiarelli et al., 2018; Sharifuldin et al., 2016) calculated LOD and LOQ values for quercetin of 0.1 and 0.3 mg/L, respectively, by analyzing red wine as a matrix. Another study determined the LOD and LOQ of quercetin in a traditional medicinal plant as

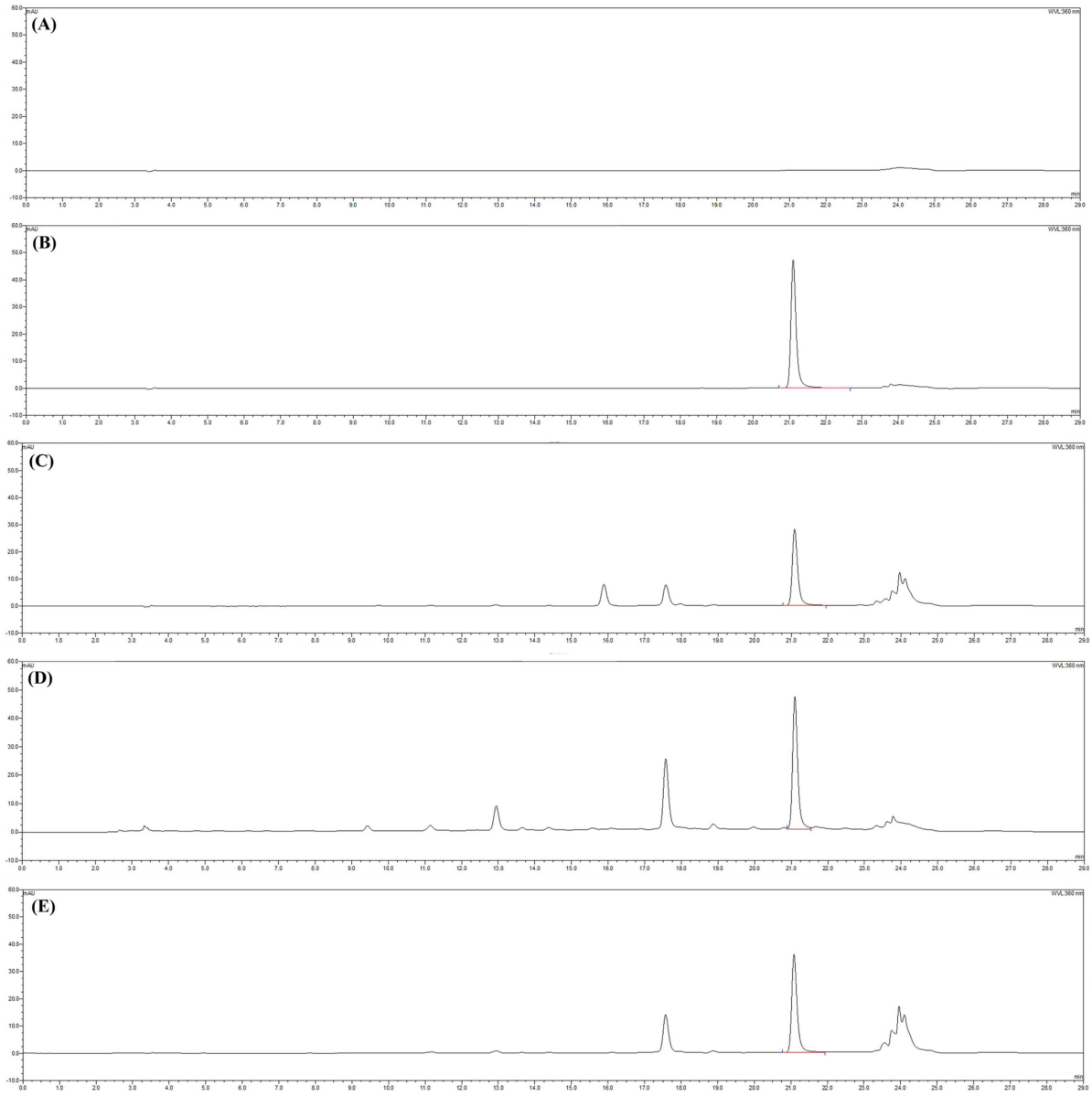

Fig. 1. Chromatograms of the blank (A), 10 mg/kg quercetin standard solution (B), a leached tea (C), a liquid tea (D), and a processed product (E)

Table 1. Calibration parameters of quercetin

Parameters	Beverage	Candy	Sausage
Regression coefficient (r^2) (Mean±SD)	1.0000±0.0000	0.9999±0.0001	0.9998±0.0002
Slope (Mean±SD)	0.822±0.010	0.788±0.010	0.805±0.010
Intercept (Mean±SD)	0.037±0.036	0.254±0.069	0.027±0.075
LOD (mg/kg)[1)]	0.15	0.29	0.31
LOQ (mg/kg)[2)]	0.44	0.87	0.93
Matrix effect (%)	0.19	3.48	3.24

[1)]Limit of detection, [2)]Limit of quantification

Table 2. Accuracy, precision, and relative uncertainty results of the proposed HPLC-UV method for quercetin quantification

Samples	Fortified concentration (mg/kg)	Intra-day[1)]			Inter-day[2)]			Relative uncertainty (%)
		Accuracy[3)] (%)	Precision (RSD%)	HorRat (r)[4)]	Accuracy (%)	Precision (RSD%)	HorRat (r)[5)]	
Beverage	0.5	86.7±2.4	2.8	0.15	88.2±2.7	3.0	0.17	12.7
	2.5	90.0±0.9	1.0	0.07	89.7±1.1	1.2	0.09	4.4
	10	94.0±1.3	1.4	0.12	93.6±1.5	1.6	0.14	2.3
Candy	0.5	87.6±0.8	0.9	0.05	88.9±1.7	1.9	0.11	8.7
	2.5	94.0±0.7	0.7	0.05	91.0±2.5	2.7	0.19	2.6
	10	102.0±1.3	1.3	0.12	101.5±1.7	1.6	0.14	1.9
Sausage	0.5	84.3±0.8	1.0	0.05	85.0±1.2	1.4	0.08	14.2
	2.5	92.2±1.5	1.6	0.11	93.1±1.6	1.7	0.12	4.1
	10	91.7±1.8	1.9	0.17	92.5±1.1	1.2	0.10	2.6

[1)]Analysis was conducted six times/day, [2)]Analysis was conducted three times on three days, [3)]Average±SD
[4)]HorRat ratio for intra-day repeatability [5)]HorRat ratio for inter-day repeatability

0.1581 and 0.4792 μg/mL, respectively.

The matrix effect values were 0.19% for beverage, 3.48% for candy, and 3.24% for sausage. The matrix effect was within ±20% based on the validation parameters and criteria of the European Commission (Sante, 2015). It confirmed that there was almost no effect of the matrix. However, to achieve more accurate and precise analysis results, validation was performed by using a matrix-matched calibration curve in this study.

The intra-day and inter-day accuracy and precision are shown in Table 2. The recovery results obtained by spiking 0.5, 2.5, and 10 mg/kg were 84.3-102.0% (intra-day) and 85.0-101.5% (inter-day). RSD (%) was 0.7-2.8% (intra-day) and 1.2-3.0% (inter-day). These results were within the acceptable range based on the AOAC validation guideline (AOAC, 2016). In addition, the HorRat (r) values were 0.05 to 0.17 for intra-day and 0.08 to 0.19 for inter-day. The measurement uncertainty was calculated based on the recovery assays of each sample in this study. The calculation process was performed considering uncertainty factors related to quercetin analysis, such as *uSSS*, *uSP*, *uCal*, and *uRP*. As shown in Table 2, compared with the analysis results, the relative uncertainty was 2.3-12.7% in beverage, 1.9-8.7% in candy, and 2.6-14.2% in sausage, respectively. The result satisfied the CODEX criteria (<22%) (Codex Alimentarius Commission, 2008). In addition, the contributions of each factor to the uncertainty of the final results are shown in Fig. 2. There was no significant difference in *uRP*, *uSP*, and *uSSS* when the factors affecting the uncertainty were calculated for the spiking concentration of each sample. However, as the concentration of added quercetin was lowered, it was confirmed that the uncertainty of *uCal* was 9.87% in beverage, 6.95% in candy, and 12.28% in sausage. Therefore, a higher proficient skill of the researcher is required for the calibration curve at the lowest concentration of sample analysis.

Inter-laboratory validation

Recovery assays were conducted in three laboratories for beverage containing quercetin, and the results were compared. Ranges of 91.75-95.89% in Lab A, 91.66-97.71% in Lab B, and 90.34-94.60% in Lab C were obtained (Table 3). Based on the AOAC validation guideline for each concentration (AOAC, 2016), the recovery rate determined in each laboratory was acceptable. Moreover, the RSD% was 1.16-1.53%, which satisfied the acceptable range for reproducibility, and the accuracy and precision of the proposed analytical method were validated.

Application

To confirm the applicability of the proposed HPLC method, it was used to analyze various products distributed in Korea, including leached teas, onion skins, liquid teas, and processed products, such as functional food and a tablet. The results are shown in Table 4. Leached teas, onion skins, liquid teas, and processed products

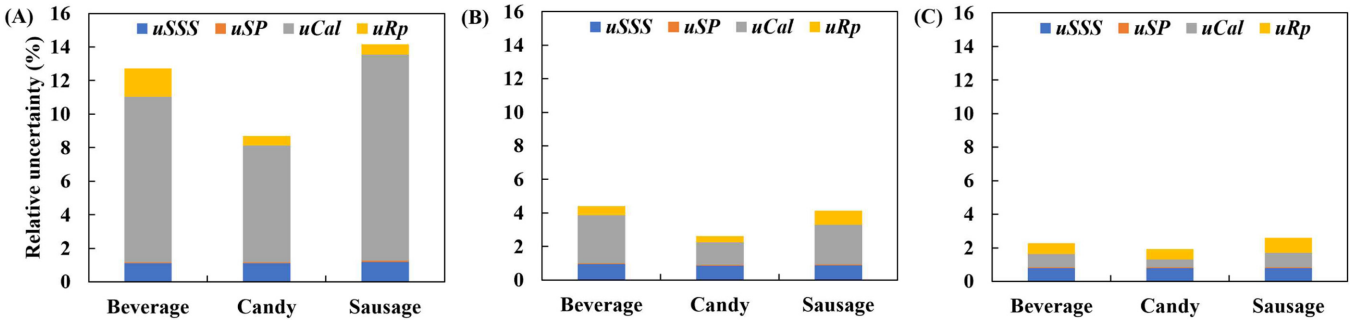

Fig. 2. Measurement uncertainty contributions to the expanded uncertainty in the determination of quercetin in beverage, candy, and sausage by the proposed HPLC-UV method (0.5 mg/kg spiking (A), 2.5 mg/kg spiking (B), and 10 mg/kg spiking (C))

Table 3. Inter-laboratory reproducibility results of recovery for quercetin

Sample	Fortified concentration (mg/kg)	Recovery±SD (%)			Average±SD (%)	RSD (%)
		Lab. A	Lab. B	Lab. C		
Beverage	0.5	91.75±0.54	91.66±1.52	90.34±0.18	91.25±1.06	1.16
	2.5	95.89±0.46	97.35±1.16	94.40±0.11	95.88±1.42	1.48
	10	95.21±0.32	97.71±0.51	94.60±0.28	95.84±1.46	1.53

Table 4. Concentration (mg/kg) and the range of quercetin in foods

Food category	Number of sample	Range		Average con. (mg/kg)
		Min con. (mg/kg)	Max con. (mg/kg)	
Leached tea	9	3,762.71	11,177.94	7,175.83
Onion skin	5	3,319.50	7,143.83	5,872.16
Liquid tea	4	2.38	46.62	21.85
Processed product	4	6.68	464,103.08	123,910.71

*All experiments were carried out in three replicates.

contained quercetin concentrations of 7,175.83 mg/kg (3,762.71-11,177.94 mg/kg), 5,872.16 mg/kg (3,319.50-7,143.83 mg/kg), 21.85 mg/kg (2.38-46.62 mg/kg), and 123,910.71 mg/kg (6.68-464,103.08 mg/kg), respectively. These findings were comparable to the literature data. For example, for the outer layers of red onion, the quantities of quercetin were 5,110 mg/kg (Prakash et al., 2007) and 2,990 mg/kg (Albishi et al., 2013). Furthermore, the food with the highest content among the collected samples was the processed product (ingredient: 50% quercetin as a food additive encapsulated in digestion-resistant maltodextrin as the carrier). We confirmed that the proposed analytical method was suitable for quantifying quercetin in various foods.

Conclusion

In this study, an HPLC-UV method for analyzing quercetin in foods was validated for specificity, linearity, precision, and accuracy. The total run time was 29 min. Method validation data have proved the method to be compliant with standard validation guidelines. Additionally, the most influential factors contributing to the measurement uncertainty were assessed, which confirmed the reliability of the results of this analysis. Furthermore, the applicability of the proposed method for quantifying quercetin in various commercial products, such as teas and beverages, was demonstrated. This method is suitable for rapid and routine analysis of quercetin in various food products.

Acknowledgment

This research was supported by a grant (19162MFDS012) from Ministry of Food and Drug Safety in 2021.

Conflict of Interest

The authors declare no conflict of interest.

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