Rapid LC-MS/MS Determination of Hesperidin in Fermented Tea Prepared from Unripe Satsuma Mandarin (Citrus unshiu) Fruits and Third-crop Green Tea (Camellia sinensis) Leaves

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For improving quality control in the fermented tea production process and advancing the corresponding food labeling with function claims, a rapid and robust hesperidin analysis method using LC-MS/MS with the sample dilution approach was developed by following internationally accepted criteria of the Association of Official Analytical Chemists (AOAC). The linear correlation coefficient (r²) of the regression line was 0.9997 in the concentration range of 0.025 - 2.5 mg/L. The matrix effect evaluated using regression line slope values was negligible. The recovery rate of 100.7% indicated improved trueness. The performance of the newly developed method in determining the hesperidin content of fermented tea samples did not significantly vary from that of a well-established, conventional method. The HorRat values of intra- and inter-laboratory reproducibility studies were both within the acceptable range, indicating sufficient accuracy of the newly developed method according to the AOAC criteria.

Keywords Sample dilution, ultra-performance LC-MS/MS, hesperidin, flavanone glycosides, fermented tea, third-crop green tea leaves, unripe satsuma mandarin fruits, inter-laboratory validation study

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Introduction

Flavored teas are popular beverages consumed worldwide. Various kinds of flavored or fruit teas have become popular in many European countries due to their fragrance, therapeutic application, and lower content of caffeine, which could inhibit calcium absorption. The flavors are produced by supplementing the tea leaves (Camellia sinensis) with various herbs and dry fruits, usually during the last processing stage before the packing. Varieties of “fermented mixed teas” have been recently developed in Nagasaki as a new type of flavored tea. They are prepared from a herbal medicinal product, such as camellia leaves, loquat leaves, or unripe satsuma mandarin (Citrus unshiu) fruits and the third-crop green tea leaves, which have a low market value, by tea rolling, which induces enzymatic oxidation of the green tea catechins to generate polyphenols similar to those of black tea, including theaflavins and theasinensins. Although the green tea leaves have a relatively low polyphenol oxidase activity, the herbal medicinal products possess a much stronger polyphenol oxidase activity, which effectively produces these black tea polyphenols.

Hesperidin, a flavanone glycoside of hesperetin, is abundant in the fruits of citrus trees. It is composed of the insoluble hesperetin moiety and a hydrophilic rutinose moiety. Hesperidin has been investigated for its physiological effects in humans, such as improvement of the lipid profiles and modification of the blood pressure. However, pharmaceutical application of hesperidin is limited due to its aqueous insolubility. Nakayama et al. reported the increased solubility of hesperidin by supplementing it with black tea extracts. Liquid chromatography, time-of-flight mass spectrometry, and NMR analyses revealed that the hesperidin solubility was improved by forming a stable complex from two hesperidin molecules and one theasinensin A molecule, which also supported the finding that tea polyphenols enhanced hesperidin solubility.

Satsuma mandarins can be used as a hesperidin source. Especially, unripe satsuma mandarins harvested in July contain 4 times more hesperidin than the ripe fruits harvested in December. Thus, the fermented tea prepared with unripe satsuma mandarins was expected to have enhanced health benefits due to the soluble hesperidin. Indeed, a recent study suggests that this fermented tea improves cold intolerance and fatigue in humans. However, since the hesperidin content varies greatly depending on the harvest time, it is necessary to control the tea production by monitoring the hesperidin content in the initial material and the tea product quickly and accurately. Moreover, rapid and robust analytical methods are needed for generating quantitative evidence, for advancing food labeling with function claims, which requires extensive sample analysis.

LC-MS/MS methods are suitable for analyzing target compounds in complex matrices due to their selectivity, sensitivity, and speed. Numerous separation and detection methods that use LC-MS/MS for polyphenol identification and quantification in different matrices have been recently reviewed. To date, hesperidin determination in flavored tea by LC-MS/MS has been reported, but these studies were mainly focused on comparing variations of the phenolic composition of commercial flavored tea products with longer...
running times, without considering the matrix effects that affect the quantification and method reproducibility.\textsuperscript{17} Another study established a rapid quantification method of multiple classes of phenolics, including hesperidin, in green tea,\textsuperscript{18} but the matrix effect was not considered during method development.

In our previous studies, a sample dilution step and low injection volume for LC-MS/MS analysis reduced the matrix effect without laborious laboratory techniques in spinach.\textsuperscript{19} In another study, a simple method for simultaneous determination of 32 phenolic compounds, including hesperidin, was optimized and validated using the dilute-and-shoot approach for honeydew honey samples.\textsuperscript{20}

Here, we applied the sample dilution approach for rapid and robust hesperidin determination in fermented tea using LC-MS/MS. In this validation study, we tested the new method and evaluated the recovery rate, conducted a comparison with a conventional method, and performed intra- and inter-laboratory tests according to international guidelines.\textsuperscript{21–25}

**Experimental**

**Reagents and chemicals**

LC/MS-grade ultrapure water, HPLC-grade methanol, and dimethyl sulfoxide were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). LC/MS-grade acetonitrile and formic acid (elucent additives for LC/MS) were purchased from Honeywell Fluka Chemicals (Morristown, NJ). Hesperidin standard for analysis and chromatography grade was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Fermented tea material and fermented tea sample**

The fermented tea sample used in this study was provided by Aliment Industry Co., Ltd. (Yamanashi, Japan). The methodology of fermented tea production has been described elsewhere.\textsuperscript{2} Briefly, the second- or third-crop tea leaves (heat-treated) and unripe satsuma mandarin fruits (sliced) were mixed at the ratio of 3 to 1 and kneaded for 20 min.\textsuperscript{2} The resulting fermented tea was heated and prepared (the fermented tea material) before adding dextrin (as a binder). Then, 0.6% (w/w) guar gum solution was sprayed onto the mixed fermented tea material, which was further processed to generate a granulated form, the fermented tea sample, using the Aliment Industry laboratory equipment. Thus, the granulated form of the fermented tea material is the fermented tea sample, consisting of powdered fermented tea leaves (74.2%), dextrin (25.6%), and guar gum (0.27%). The fermented tea sample was stored at -80°C until use. The fermented tea sample was used in the validation study unless otherwise indicated. Occasionally, the fermented tea material was also used to assess the applicability of the newly developed method for variations in the sample composition.

**Preparation and extraction of hesperidin from the fermented tea sample**

Ten milligrams of fermented tea sample was weighed into a 50-mL tube and dissolved in 10 mL of a solvent mixture of dimethyl sulfoxide and methanol (50:50 v/v; extraction solution). Then, for 10 min, the tube was placed into an ultrasonic bath (KAIJO Sono Cleaner D400) using the settings of 38 kHz and 400 W at ambient temperature (28°C) to promote hesperidin extraction. The resulting sample extract solution was filtered through a 0.2-μm polyvinylidene difluoride membrane filter (Whatman™ GD/X™ 25 syringe filter; Florham Park, NJ) and diluted (1:100) in a solution composed of water with 0.1% formic acid and acetonitrile/methanol (50:50 v/v) supplemented with formic acid (final concentration 0.1%) that were mixed at a ratio of 7:3 (dilution solution). Then, 1 μL of the diluted sample was injected into a ultra-performance LC-MS/MS system.

**Quantification of hesperidin content in the fermented tea sample**

We determined the hesperidin content using a liquid chromatography-tandem quadrupole mass spectrometry system (Waters Acquity UPLC H-Class Xevo for HPLC and TQ-S micro for MS/MS; Milford, MA). Samples were injected into a HALO C18 column (2.7 μm, 2.1 × 75 mm) kept at 40°C. The binary solvent gradient conditions were as follows. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile/methanol (50:50 v/v) supplemented with formic acid (final concentration 0.1%). The column was eluted at a flow rate of 0.4 mL/min, and the gradient elution profile was initiated with 70% A and 30% B for 2.5 min. The proportion of mobile phase A was then decreased to 10%, increasing mobile phase B to 90%. Finally, the initial mobile phase proportions were restored within 4 min to equilibrate the column for a further 2.6 min. Prior to the MS/MS analysis, in-line UV detection was carried out at 285 nm.\textsuperscript{31} The samples were then analyzed using the electrospray ionization (ESI) probe in positive ion mode. Nitrogen source gas was used at a flow rate of 500 L/h (desolvation). The ESI probe temperature was maintained at 600°C. Source conditions were set to 0.20 kV (capillary) and 30 V (cone). The hesperidin content in samples was identified and quantified using the multiple reaction monitoring mode with the following transition: hesperidin m/z 610.97 → m/z 302.95.\textsuperscript{26} The relative content of hesperidin was determined based on the analytical area of sample peaks using an automated peak annotation program in the Waters MassLynx4.1 software (Milford, MA).

**Linearity, limit of detection, and limit of quantification**

Hesperidin standard stock solutions (100 mg/L) and its working solutions (5, 10, 25, 50, 75 mg/L) were prepared using a diluent composed of dimethyl sulfoxide and methanol (50:50 v/v), and hesperidin calibration standards were freshly made by diluting the stock solution with the dilution solution described in the previous section. Calibration curves were constructed by plotting the peak area values versus the concentrations of the hesperidin standards (0.025, 0.05, 0.1, 0.25, 1.0 and 2.5 mg/L for linearity tests, and 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L for hesperidin quantification), and linear regression was used to generate a regression line. The limit of detection (LOD) and quantification (LOQ) were estimated based on the standard deviation of relative intensity and slope according to internationally recognized guidelines.\textsuperscript{27,28}

**Test for efficiency of ultrasonic extraction**

The method for testing the ultrasonic extraction efficiency is described in Supporting Information.

**Evaluation of the matrix effect**

For absolute calibration, six concentrations (0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L) of hesperidin standard were prepared in the dilution solution. For matrix calibration, 900 μL of 100-fold diluted fermented tea sample or fermented tea material extract (n = 5) in the dilution solution were spiked with 100 μL of hesperidin standards (0, 5 and 10 mg/L). Matrix effect was evaluated using the following equation:\textsuperscript{20}

\[
\text{Matrix effect (\%)} = (1 - \text{slope in matrix calibration/slope in absolute calibration}) \times 100
\]
Fermented tea sample 10 mg (± 5%) + Dimethyl sulfoxide and methanol (50:50 v/v)  
50 mL tube  
Ultrasound extraction  
10 min, ambient temperature  
Filtration  
0.2-μm polyvinylidene difluoride membrane filter  
Dilution  
0.1%(v/v) formic acid in water and 0.1%(v/v) formic acid in acetonitrile/methanol (50:50 v/v) (7.3 v/v)  
LC-MS/MS  
1 µL injection  

Fig. 1 Flowchart of distributed standard operating procedure.

Spike-and-recovery experiment
Ten milligrams of fermented tea sample was spiked with 2.0 mL of hesperidin standard (250 mg/L) at a level of 161.3% of the estimated content (0.31 mg/10 mg) of hesperidin in the un-spiked samples. For the fermented tea material, the standard was used to spike at a level of 79.4% of the estimated content (0.63 mg/13.5 mg). The recovery rate was calculated from the mean values of the obtained values (n = 3) using the following equation:

\[ \text{Recovery} (\%) = \left( \frac{\text{spiked experimental concentration} - \text{un-spiked experimental concentration}}{\text{spiked calculated concentration}} \right) \times 100. \tag{2} \]

The calculated recovery value was assessed based on the recovery rates recommended by the AOAC guidelines.\(^{22}\)

Comparison of the newly developed method with a conventional method
The fermented tea sample was prepared in five different sets of test vials. The hesperidin content was determined using a conventional method based on C18 reversed-phase HPLC column with UV-Vis detector\(^{20}\) and conducted by an independent, third-party testing laboratory to obtain an unbiased quantitative data set. A brief description of the method is provided in Supporting Information.

Homogeneity test
The fermented tea sample was used to assess homogeneity according to the IUPAC harmonized protocol.\(^{23}\) A detailed description of the calculation of the homogeneity test parameters is provided in Supporting Information and elsewhere.\(^{19}\)

Within-laboratory reproducibility
Analyses were designed to be conducted using duplicates of each fermented tea sample on two different days by three different analysts. A one-way analysis of variance was used to determine the mean square values of between-group and within-group comparisons. A detailed description of the calculation of the intra-laboratory reproducibility test is provided in Supporting Information and elsewhere.\(^{19}\)

Inter-laboratory reproducibility
We conducted a collaborative study in accordance with the instructions of the AOAC guidelines with five laboratories.\(^{25}\) All laboratories were instructed to use the same reagents and fermented tea sample in analysis. And a collaborative study was carried out as shown in Fig. 1. A detailed description of the calculation of the inter-laboratory reproducibility test is provided in Supporting Information and elsewhere.\(^{17}\)

Statistical analysis of the data
Data analyses are described in depth in the relevant sections. The statistical evaluation of linear regression using the lack of fit test and Fisher-Snedecor test was carried out on spreadsheets using Microsoft Excel 2010.\(^{31}\) Differences assessed using Student’s t-test in Microsoft Excel 2010 were considered significant at \( p < 0.05. \)

Results and Discussion

Characteristics of method performance
A representative example of hesperidin separation using a reversed-phase C18 column and optimized gradient elution conditions is shown in Fig. 2. The in-line UV detection indicated that the hesperidin standard (1.3 min in Fig. 2A) had a matching hesperidin peak in the fermented tea sample (1.3 min in Fig. 2D) with the same retention time, and their absorbance spectra with a maximum at the wavelength of 285 nm (Figs. 2B and 2E, respectively) established the identification of the peak from the sample. These peaks were detected using an optimal MRM transition for hesperidin (m/z 610.97 > 302.65) in the MS/MS detection. The peak shape and retention time of hesperidin in the fermented tea sample resembled those in the standard (Figs. 2C and 2F, respectively). The MRM transition of hesperidin using ESI operated in positive ion mode has been used elsewhere.\(^{26}\)

The analysis times provided in the related literature\(^{6,15-17}\) (running time: 15 – 30 min; hesperidin retention time: 5.84, 5.75 min) were longer than in our analysis protocol (running time: 7 min; hesperidin retention time: 1.2 min). These extended analysis times may be due to the analytical conditions that require sufficient separation to ensure selectivity for the target compounds. Since our method was focused on measuring only hesperidin, a relatively short running time and retention time were achieved. The use of a relatively short column (this study: 75 mm) may enhance the desired characteristics for a fast-throughput determination.

Linearity, limit of detection, and limit of quantification
The linearity of the hesperidin standard for our method was determined using six concentrations (\( n = 5 \)), and the parameters were compared with those obtained using in-line UV-detection (Table 1; Table S1 Supporting Information). The limit of detection (LOD) and the limit of quantification (LOQ) were derived using the standard deviation value of the intercept divided by the slope of the regression line.\(^{27,28}\) The observed LOD and LOQ values for MS/MS detection were both 15-fold lower compared with those for in-line UV-detection. The slope of the regression line for MS/MS detection was 188-fold higher than that for UV-detection. The correlation of determination \( (r^2) \) was more than 0.999 for the used concentration range (0.025 – 2.5 mg/L in the MS/MS-detection; 0.05 – 2.5 mg/L in the UV-detection).
Improvement of the peak shape and optimization of the ultrasonic extraction conditions

During preliminary experiments, we noticed that the shape of the hesperidin peak detected using LC-MS/MS was sometimes unstable when the mobile phase B was used for diluting the sample extract (Fig. S1 panel A, Supporting Information). The abnormal peak shape (peak cleavage) was substantially improved when the initial mobile phase composition of the HPLC gradient was used for preparing the dilution solution (Fig. S1 panel B, Supporting Information), which could improve the precision of the peak area estimates of the newly developed method.

Ultrasonic extraction was chosen as a simple and effective extraction method.32 To ensure improved simplicity and rapidity of the newly developed method, the ultrasonic extraction step for hesperidin in the fermented tea was optimized. Solvents used for ultrasound extraction included methanol–dimethyl sulfoxide mixture (50:50), because polyphenols are typically recovered from citrus pomace using methanol, ethanol, or aqueous mixtures of these solvents,33 and hesperidin is highly soluble in dimethyl sulfoxide.34 We tested combinations of shaking manually (20 s) and/or ultrasonic extraction (5 min or 10 min; Table S2 Supporting Information). The results suggested that 10 min of ultrasonic extraction treatment alone achieved an almost maximum recovery. These observations were further supported by an extraction efficiency (hesperidin recovery) test using a series of repeated-centrifugations and re-extractions (Table S3 Supporting Information). The data suggested that by the 2nd extraction after a total of 10 min ultrasonic treatment, more than 99% of hesperidin was recovered from the fermented tea sample. We also obtained similar values when the fermented tea material was used as starting material (Table S4 Supporting Information). Since the recovery value was within the acceptable extraction efficiency range of the AOAC guideline (92–105%),22 an ultrasonic extraction step of 10 min was applied in the newly developed method.

Method application for the fermented tea sample

The suitability of the proposed analytical method was tested by applying it to the fermented tea sample. To address matrix-related issues, the hesperidin content of the fermented tea sample was determined by both matrix calibration and absolute calibration (Table 1; Table S1 Supporting Information), and then the matrix effect was evaluated using the slopes of the two calibrations in Eq. (1).29 The UV-detection values were used as negative controls for the matrix effect in the MS/MS-detection. In the MS/MS detection, there was a 0.93% difference between the regression line slope values of the solvent and the matrix, which was similar to that in the UV-detection. The percentage difference between the mean hesperidin content of the solvent
and the matrix was 1.75% in the MS/MS detection, which was similar to that in the UV-detection, suggesting that the matrix effect of the newly developed method was negligible. Similar results were obtained when the fermented tea material was subjected to the same analysis (Table S5 Supporting Information). Unless a negligible level of matrix effect is achieved, an additional standard calibration procedure should be employed for reliable analysis, even if it is time-consuming.17 Thus, a simple quantification method for the hesperidin content in the fermented tea using LC-MS/MS has been achieved with the sample dilution approach.

Spike-and-recovery experiment
To assess method trueness, a recovery test was performed using fortified fermented tea samples (n = 3; Table 1). The fortification level was based on the innate concentration of hesperidin in the fermented tea sample (0.31 mg/10 mg). The mean recovery value was satisfactory according to the AOAC criteria22 and similar to that in the literature.17 An acceptable recovery value was also obtained when the fermented tea material was subjected to the same test (Table S6 Supporting Information).

Within-laboratory reproducibility
The hesperidin content of duplicate samples was determined on two different days by three different analysts (Table 1). The average concentration values did not differ significantly (comparisons by the percentage difference criterion22 and similar to that in the literature.17 An acceptable recovery value was also obtained when the fermented tea material was subjected to the same test (Table S6 Supporting Information).

Method-comparison test
To further evaluate method trueness, a comparison test was performed using a widely applied hesperidin determination method (CN)23 and similar to that in the literature.17 An acceptable recovery value was also obtained when the fermented tea material was subjected to the same test (Table S6 Supporting Information).

Table 2 Comparison of the performance of the newly developed method (NW) with the performance of the conventional method (CN)

| Aliquot no. | Sample 1 |   |   |
|-------------|----------|---|---|
|             | NW⁵ | CN⁶ |
| 1           | 3.33 | 3.40 |
| 2           | 3.46 | 3.47 |
| 3           | 3.46 | 3.47 |
| 4           | 3.22 | 3.43 |
| 5           | 3.44 | 3.41 |

| Content/g 100 g⁻¹ | NW⁵ | CN⁶ |
|-------------------|-----|-----|
|                   | 3.38 | 3.44 |
| RSD, %            | 3.12 | 0.96 |
| p-Value            | 0.31 |
| Mean content/g 100 g⁻¹ | 3.41 |
| Difference         | 0.06 |
| Percentage difference, % | 1.58 |

a. NW, the newly developed method.
b. CN, a conventional method.³⁰

c. Student’s t-test between NW and CN.
d. Percent difference (%) = (difference/mean) × 100.

Hesperidin content/g 100 g⁻¹

| No. of labs (c) | 5 (0) |
|-----------------|-------|
| Mean/g 100 g⁻¹  | 3.31  |
| S²/g 100 g⁻¹    | 0.10  |
| 2.8 × S²/g 100 g⁻¹ | 0.28 |
| RSDᵣ, %        | 3.03  |
| Sᵣ/g 100 g⁻¹   | 0.14  |
| 2.8 × Sᵣ/g 100 g⁻¹ | 0.40 |
| RSDᵣ, %        | 4.35  |
| PRSDᵣ, %       | 3.34  |
| HorRᵣ<sub>b</sub> | 1.30 |

a. Laboratories nos. 4 and 5 belong to the same institute, but they are spatially and financially independent and were enlisted as separate laboratories.²⁵
b. Number of laboratories remaining after the removal of the number of outliers indicated by (c).
c. d. Sᵣ: repeatability standard deviation.
e. 2.8 × Sᵣ: repeatability limit.
f. RSDᵣ: repeatability relative standard deviation.
g. Sᵣ: reproducibility standard deviation.
h. 2.8 × Sᵣ: reproducibility limit.
i. RSDᵣ: reproducibility relative standard deviation.
j. PRSDᵣ: Horwitz-predicted RSDᵣ (PRSDᵣ = 2<sup>0.1505</sup>; C = (mean/g 100 g⁻¹) × 10⁻²).
k. HorRᵣ: RSDᵣ/PRSDᵣ (The limit for performance acceptability is 0.5 – 2.0).²⁵

Hesperidin content/g 100 g⁻¹

| No. of labs (c) | 5 (0) |
|-----------------|-------|
| Mean/g 100 g⁻¹  | 3.31  |
| S²/g 100 g⁻¹    | 0.10  |
| 2.8 × S²/g 100 g⁻¹ | 0.28 |
| RSDᵣ, %        | 3.03  |
| Sᵣ/g 100 g⁻¹   | 0.14  |
| 2.8 × Sᵣ/g 100 g⁻¹ | 0.40 |
| RSDᵣ, %        | 4.35  |
| PRSDᵣ, %       | 3.34  |
| HorRᵣ<sub>b</sub> | 1.30 |

a. Laboratories nos. 4 and 5 belong to the same institute, but they are spatially and financially independent and were enlisted as separate laboratories.²⁵
b. Number of laboratories remaining after the removal of the number of outliers indicated by (c).
c. d. Sᵣ: repeatability standard deviation.
e. 2.8 × Sᵣ: repeatability limit.
f. RSDᵣ: repeatability relative standard deviation.
g. Sᵣ: reproducibility standard deviation.
h. 2.8 × Sᵣ: reproducibility limit.
i. RSDᵣ: reproducibility relative standard deviation.
j. PRSDᵣ: Horwitz-predicted RSDᵣ (PRSDᵣ = 2<sup>0.1505</sup>; C = (mean/g 100 g⁻¹) × 10⁻²).
k. HorRᵣ: RSDᵣ/PRSDᵣ (The limit for performance acceptability is 0.5 – 2.0).²⁵

Homogeneity test
Homogeneity of the fermented tea sample was confirmed according to the applicable international guideline.²³,²⁴ The data set and parameters are shown in Table S7 (Supporting Information). Therefore, the fermented tea sample was used for the intra- and inter-laboratory reproducibility studies.

Inter-laboratory reproducibility
Our inter-laboratory reproducibility study was limited to five participating laboratories that fulfilled the requirements for the expensive specialized equipment, which included an LC-MS/ MS system using an ESI probe. A set of five laboratories is considered acceptable in exceptional cases.²⁵ The analytical instruments, columns, regression lines, regression coefficients, retention times of the hesperidin peaks, and MRM transitions used by each participating laboratory are summarized in Tables S8 – S10 (Supporting Information). Two participating laboratories, nos. 4 and 5, belonged to the same institute, but their spatial and financial independence were the main criteria for defining them as separate laboratories.²⁵ Remarkably, the slope value of the calibration line reported by laboratory no. 1 was more than...
Conclusions

We focused on hesperidin determination in the fermented tea sample. Data from the validation study suggested that the total processing time of the newly developed method, including extraction and analysis, was less than 30 min per sample, which was approximately 7-fold less time-consuming, compared with that of a well-established, conventional method. A sample dilution step and low injection volume, along with a negligible matrix effect, are other advantages compared to the related methods.3,15-17 To our knowledge, this is the first report of a hesperidin determination method in green tea including flavored tea using LC-MS/MS with a negligible matrix effect (−1.13%). Moreover, both repeatability and reproducibility, along with the recovery rate, were within the acceptable range for a validated method according to the AOAC criteria, which represented a significant improvement over related methods.10

The fermented tea sample used in this study was a granulated form of tea that consisted of satsuma mandarin fruits and green tea leaves, including binders (dextrin and guar gum).12 Because all our analysis results for extraction efficiency, matrix effect, and spike-and-recovery tests were also within the acceptable range when the fermented tea material was used, this method should be fully applicable for the fermented tea material. Our newly developed method could also be used for various other flavored teas prepared with citrus species.

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Supporting Information

Supporting Information includes Fig. S1 (improvement of the peak shape), Tables S1 – S6 (validation study results), Table S7 (homogeneity test results), and Tables S8 – S11 (instrument summary, regression lines, regression coefficients, retention times of the hesperidin peaks, and MRM transitions used in the inter-laboratory study). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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