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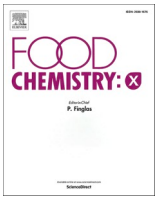

Identification of marker compounds in fermented *Benincasa hispida* and validation of the method for its analysis

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ABSTRACT

Fermentation is a process that improves health functionality by inducing the production and increase of bioactive compounds. In this study, to standardize the fermentation process for *Benincasa hispida*, marker compounds that are increased or produced during fermentation were identified based on UPLC-QTOF-MS/MS. Analysis method verification and content analysis were conducted using HPLC-PDA. The marker compounds produced or increased in content were identified as 2-furoic acid, 2,3-dihydroxybenzoic acid, and rubinaphthin A by comparing their retention times, UV and MS spectra, and molecular formulas with those reported in previous studies. In addition, the increase in the content of the marker compounds by fermentation was confirmed, and the analytical method was validated by measuring its specificity, linearity, limit of detection and quantitation, precision, and accuracy. These results suggest that the developed fermentation process, marker compound identification, and verified analysis method can be applied to develop potential functional food ingredients from fermented *B. hispida*.

1. Introduction

Benincasa hispida (wax gourd) is an annual vine belonging to the Cucurbitaceae family native to tropical Asia. *B. hispida* is a vegetable widely distributed in the dry regions of tropical central and Asian countries, such as Japan, China, Korea, and India (Mohammad, Anwar, Mehmood, Hamid, Muhammad & Saari, 2019). It is called white gourd, winter melon, ash gourd, or wax gourd because the surface of the mature fruit is covered with a characteristic white wax substance (Pradhan, Nandi, Das, Sarkar, Sahu & Patnaik, 2018). *B. hispida* usually uses fruits weighing less than 10 kg, and fruits and seeds are present in the Korean food material database (Ministry of Food and Drug Safety Korea, 2021). *B. hispida* is used for medicinal and nutritional purposes, especially in Asian countries; as a food material, it is limited to being consumed boiled or raw (Harmayani et al., 2019). Therefore, it is necessary to develop high-value-added functional foods using *B. hispida* as a functional ingredient. Oriental medicine has been reported to improve blood cholesterol levels, obesity, and diabetes. In particular, improvements in diuretics and constipation have been reported by strengthening the movement function of the large and small intestines (Islam et al., 2021). The pulp of *B. hispida* contains uronic acid, ß-sitosterin, vitamins, carotenes, saccharides, glycosides, flavonoids, and volatile oils as bioactive compounds (Tamilarasan, Rathinam, Ayyasamy, Balasubramaniyan, Munusamy & Parameswaran, 2022). In addition, pharmacological studies of *B. hispida* have reported antimicrobial, hypolipidemic, anti-inflammatory, nephroprotective, anti-diabetic, diuretic, antiasthmatic, analgesic, antioxidant, and central nervous system stabilization (Alsaadia & Abass, 2020; Chaitali,Rao,Vikhe, 2022). It has been reported that the functional and biological efficacy of *B. hispida* is due to the high content of physiologically active substances such as triterpenes, phenols, sterols, and glycosides (Alsaadia & Abass, 2020). Based on these various physiological activities, the possibility of developing functional foods using *B. hispida* fruit is suggested.

Fermentation is a process in which microorganisms change organic matter through enzymatic action, breaking down large molecules into small molecules and involving molecular oxidation/reduction

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mechanisms (Sun, Shahrajabian & Lin, 2022). In the fermentation process, microorganisms use sugar to produce alcohol and organic acid CO_2 as fermentation products and increase digestion and absorption in the body through decomposition into small molecules (Sharma, Garg, Kumar, Bhatia & Kulshrestha, 2020). The production of organic acids inhibits the growth of pathogenic bacteria and improves taste, flavor, and texture (Bangar, Suri, Trif & Ozogul, 2022). Furthermore, it improves health by inducing the production of bioactive compounds (Septembre-Malaterre, Remize & Poucheret, 2018). Bioconversion is a type of fermentation process that uses microorganisms and enzymes and refers to a technology that produces and manufactures desired products from precursors. The bioconversion process uses the selectivity of microorganisms or enzymes for substrates to induce biological changes such as increased content and absorption of bioactive components through structural changes in existing materials (Kaur, Singh & Singh, 2023). The fermentation microorganism used in this study is *Bacillus subtilis*, which is a representative fermentation microorganism widely used in industries because it produces proteolytic enzymes and is stable to heat and pH (Kimura & Yokoyama, 2019; Miyazawa, Abe, Bhaswant, Ikeda, Higuchi & Miyazawa, 2022). Microorganisms of the genus *Bacillus* produce various secondary metabolites through useful enzymes such as amylase, protease, cellulase, and glucosidase and are widely used to improve the properties of food ingredients (Woldemariam et al., 2020). However, fermentation is difficult to standardize because microbial diversity, characteristics and amount of raw material, fermentation condition (time, temperature, and pH) affect microbial growth and activity (Tachie, Onuh & Aryee, 2024). In this study, *B. subtilis* isolated from Cheonggukjang, a traditional Korean fermented food, was used for fermentation. The fermentation process was optimized by evaluating the cytotoxicity, immune function improvement, and antioxidant efficacy of the material under various conditions based on microbial diversity, fermentation conditions, and number of viable bacteria by period (data not shown). In addition, to standardize fermented *B. hispida* extract (FBE) developed under optimal fermentation conditions, this study performed identification of marker compounds, and development and verification of analysis methods.

B. hispida has been used as a food ingredient and traditional medicine for thousands of years (Islam et al., 2021). Although various studies have been conducted on the physiological functionality of *B. hispida*, there has been no research on the development of functional foods (Choi et al., 2022; Choi et al., 2023). The fruits of *B. hispida* is suitable for fermentation by *B. subtilis* as a potential source of polysaccharides (Wang et al., 2022). We attempted bioconversion using fermentation to increase the physiological functionality of the *B. hispida*,and confirmed an increase in immunomodulatory effect in mice administered FBE rather than *B. hispida* extract (BHE) (Choi et al., 2024). To develop FBE into functional food ingredients, standardization through analysis of changes in bioactive compounds following bioconversion, selection, and analysis of marker compounds should be performed. In this study, the marker compounds required for the standardization of the fermentation process and product were identified to develop FBE as a functional food. Standardization is a manufacturing process norm used throughout the process to maintain a consistent quality from the raw materials to the final products (Rupasinghe, Davis, Kumar, Murray & Zheljazkov, 2020). In addition, a standardization method based on the measurement of marker compounds is most used. Marker compounds should be selected considering the specificity of components whose content varies depending on the raw materials or manufacturing methods and the representativeness of components involved in functions due to differences in content or presence/absence (Balekundri & Mannur, 2020). Busuioc et al. (2020) reported that gallic acid is a major bioactive component of *B. hispida*. However, because gallic acid is a representative polyphenol contained in many plants, it is not suitable for use as a marker component because of its lack of specificity and representativeness. In our previous study, 2-furoic acid (2-FA) was identified as a marker compound for BHE, a high-performance liquid chromatography-photodiode array (HPLC-PDA) analysis method was developed for its identification (Choi et al., 2021).

In this study, an analysis method developed and validated in the BHE matrix was applied considering specificity, representativeness, and convenience. The bioactive compounds produced in the bioconversion process were identified, and changes in content were confirmed for the standardization of FBE. In addition, the analysis of marker compounds for standardization requires accurate and precise analytical methods, and scientific validity and reliability must be proven. Therefore, the possibility of developing functional food ingredients with FBE was suggested by validating the analysis method through the measurement of specificity, linearity, accuracy, precision, detection limit, and quantification limit of the marker compounds.

2. Materials and methods

2.1. Chemicals and standards

Chemical standards 2-FA (CAS:88–14-2; ≥ 98 %), 2,3-Dihydroxybenzoic acid (2,3-DHBA; CAS:303–38-8; ≥ 99 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rubinaphthin A (RUA; CAS:448962–05-8; ≥ 98 %) was purchased from ChemFaces (Wuhan, Hubei, China). Trifluoroacetic acid (≥99 %; J.T. Baker, Philipsburg, NJ, USA) was used as mobile phase A at 0.1 % in distilled water, and acetonitrile (ACN; ≥ 99 %) was purchased from Sigma-Aldrich as mobile phase B for HPLC analysis. Formic acid (≥98 %; Junsei, Tokyo, Japan) was diluted with distilled water (mobile phase A) and ACN (mobile phase B) at 0.1 % for ultra-performance liquid chromatography (UPLC) analysis.

2.2. Sample preparation

B. hispida harvested from Chungbuk, Goesan, Korea in August 2020 was provided by Haram Co., Ltd. (Chungbuk, Korea). *B. hispida* was identified by Dr. Ju-Hyun Cho of the Haram Central Research Institute (Chungbuk, Korea). *B. hispida* was fermented with *B. subtilis* CJH 101 using the following fermentation system: the pulp of the *B. hispida* fruit, except for the peel and seeds, was used. The fermentation medium was prepared by mixing pulverized pulp of *B. hispida* and distilled water 1:1 (*w/w*), and the medium was sterilized in an autoclave at 121 °C for 15 min. *B. subtilis* CJH101 was incubated in 100 mL of nutrient (BD cell, Seoul, Korea) broth at 30 °C for 15 h to activate and used as a spawn. After inoculating 3 kg of fermentation medium with 100 mL of spawn (1.9×10^8 CFU/mL), it was fermented at 30 °C for 72 h in an incubator (FMT ST-S, Fermentech, Korea). The ferment was extracted with distilled water at 120 °C for 4 h and freeze-dried (Labconco, Kansas City, MO, USA) to obtain FBE (HR1901-BS). The freeze-dried FBE sample (400 mg) was mixed with 10 mL of distilled water and sonicated for 30 min. The solution was filtered using a 0.22 μm polyvinylidene fluoride filter prior to use.

2.3. Characterization of marker compounds using UPLC-quadrupole time of flight (QTOF)-mass spectrometry/mass spectrometry (MS/MS)

LC-MS analysis was performed on a Waters Acquity UPLC I-Class system (Waters Co., Milford, MA, USA) coupled with a Waters Xevo G2 QTOF MS system (Waters Co.) at the Chuncheon Center of Korea Basic Science Institute (KBSI). Chromatographic separation was performed with a Waters Acquity UPLC BEH C18 (150 mm × 2.1 mm, 1.7 μm) maintained at 40 °C, and the injection volume was set at 2 μL. The mobile phase was composed of two mobile phases (A:0.1 % (*v/v*) formic acid in distilled water; B: 0.1 % (*v/v*) formic acid in acetonitrile) with gradient elution (10–90 % B, 0–14 min) at a flow rate of 400 μL/min. QTOF-MS analysis was performed in electrospray ionization (ESI) negative ion mode in a continuum format. MS/MS ion patterns were scanned within the mass range of *m/z* 100 to 1200, and the collision

energy ramp was set from 15 to 45 eV in MS^E mode. The ESI source had the following settings: the capillary and cone voltages were 2.5 kV and 45 V, respectively; temperatures of the source and desolvation gas were 120 and 350 °C, respectively; the cone and desolvation gas flows were 50 L/h and 800 L/h, respectively. To ensure reproducibility and accuracy, leucine encephalin was used as the reference compound (m/z 554.2615) at a flow rate of 5 and 200 pg/μL. The instrument was controlled MassLynx V4.1 software (Waters Corporation, Milford, USA).

2.4. HPLC instrument conditions

HPLC-PDA analysis was performed on a Waters 2695 separation module HPLC system (Waters Co., Milford, MA, USA) coupled with a Waters 996 photodiode array detector (Waters Co.). Chromatography was separated on an Osaka soda Capcell Pak C18 UG120 column (4.6 mm × 250 mm, 5.0 μm, Shiseido, Tokyo, Japan), and the column oven temperature was maintained at 30 °C. For detection, the mobile phases were 0.1 % (v/v) trifluoroacetic acid in distilled water (A) and acetonitrile (B), and the following gradient was used: 0–5 min, maintained at 90 % A; 5–15 min, linear from 90 to 70 % A; 15–20 min, linear from 70 to 90 %; 20–25 min, maintained at 90 % A. The mobile phase was filtered using a 0.45 μm membrane filter and degassed prior to use. Marker compounds were monitored at 254 nm.

2.5. Validation of the HPLC analytical method

The HPLC analysis method was validated according to the procedure presented in the International Conference on Harmonization (ICH) guidelines (ICH Expert Working Group, 2022). The analytical method for determining 2-FA, 2,3-DHBA, and RUA in BHE and FBE was validated in terms of specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy.

2.5.1. Specificity and confirmation of identity

Confirmation of the identity and specificity of the compound was ensured through HPLC separation and UV and MS spectra. LC peak separation, retention times (RTs), and spectra were evaluated, standard products were spiked, and signal changes were observed. The resolution is a measure of the degree of separation of two peaks, and completely separating the peaks means that the resolutions have resolution (Rs) > 2 (European Commission, 2017), as calculated by the following formula: $Rs\ (\%) = [2(t_{R2} - t_{R1}) / (W_1 + W_2)]$, where t_R is the RT, and W is the peak width. Unique ion fragmentation identified by the assigned qualifier and quantifier ions was monitored to ensure the specific identification of the compounds. This was confirmed by the results of the samples comparing the RTs and ion fragmentation of standard compounds.

2.5.2. Linearity, LOD, and LOQ

Calibration curves were generated by diluting the stock solution with methanol (≥99 %; J.T. Baker) at a seven-point concentration (1.56–100 μg/mL). Sensitivity was determined by the change in the response of a measuring instrument divided by the corresponding change in the stimulus, which was the slope of the calibration function. The LOD and LOQ of the marker compounds (2-FA, 2,3-DHBA, and RUA) were calculated using the following formula based on the slope and the standard deviation: $LOD = 3.3(\sigma/S)$; $LOQ = 10(\sigma/S)$, where σ is the mean standard deviation and S is the slope of the calibration curve.

2.5.3. Precision and accuracy

Accuracy and precision were measured in two matrices in which standard solutions were spiked with BHE and FBE at 12.5, 25, and 50 μg/mL concentrations. All samples were analyzed in triplicate (intraday) on three different days (interday). Precision was determined using the relative standard deviation (RSD). Accuracy was obtained by calculating the recovered concentration for the spiked concentration using the following formula: $recovery\ (\%) = [(C_f - C_u) / C_a] \times 100$, where C_f is the concentration of the spiked sample, C_u is the concentration of the sample, and C_a is the concentration of the standard.

2.6. Quantification of targeted marker compounds

Each analyte peak was identified by comparing the PDA spectrum with the RT of the standards. The standard calibration curve was determined using a seven-point concentration (0.10–6.25 μg/mL) of the standard mixture. The amounts of marker compounds in the three batches of BHE and FBE were calculated using the prepared standard calibration curve.

2.7. Standards and working solutions

All stock solutions of 2-FA, 2,3-DHBA, and RUA were prepared at 1 mg/mL in methanol. A working standard mixture was used for standard calibration curve generation and sample spiking by mixing the appropriate concentrations of stock solutions and methanol.

3. Results and discussion

3.1. Identification of marker compound in FBE

3.1.1. HPLC-PDA analysis

Standardizing functional food ingredients is an essential process that must be prioritized along with their safety and functionality. Standardization in functional food development must satisfy the criteria for standardization of raw materials, manufacturing processes, and analytical methods (Alongi & Anese, 2021). In addition, as standardization is performed through the analysis of marker compounds, the identification and development of analytical methods for marker compounds must be performed. The most well-known bioactive phytochemical *in B. hispida* is gallic acid (Busuioc et al., 2020). Analysis of gallic acid present in BHE and FBE showed the same RT as the gallic acid standard compound. However, it cannot be identified as gallic acid because the maximum absorption wavelength region is different in the PDA spectrum (Fig. S1). To standardize *B. hispida* raw materials, identification of 2-FA as marker compound and analytical method development were performed in previous research (Choi et al., 2021). In this study, the 2-FA analysis method was applied to identify the marker compounds for FBE, considering the continuity of standardization (*B. hispida* raw materials, fermentation process, and FBE). As a result of fermentation, an increased peak area is observed for 2-FA and new peaks are confirmed in the 14 and 17 min regions (Fig. S2). The newly generated peak was confirmed as a marker compound that can be applied to the standardization of the fermentation process, and unknown compounds were identified.

3.1.2. UPLC-QTOF-MS/MS analysis

UPLC-QTOF-MS/MS provides high mass accuracy, sensitivity, and information for compound structural analysis, allowing for screening and unambiguous identification of unknown compounds. As shown in Fig. 1a, for UPLC-QTOF-MS/MS profiling of BHE and FBE in 70 % methanol solvent, base peak intensities are confirmed in the 2.02 and 2.92 min regions. The details of the compounds identified using the m/z values of the molecular ion $[M\text{-}H]^-$, observed masses, and calculated molecular formulas are shown in Table 1. The generated compounds were identified as 2,3-DHBA and RUA by comparing the retention time and UV spectrum obtained by HPLC-PDA analysis, the MS spectrum and molecular formula obtained by UPLC-QTOF-MS/MS, and the PubChem database (2,3-DHBA, PubChem compound identifier (CID):19; RUA, PubChem CID:11760306). 2,3-DHBA shows an ion at m/z 153 $[M\text{-}H]^-$ in ESI negative mode. In addition, a fragment ion of m/z 109 $[(M\text{-}H) - 44]^-$ is observed due to the loss of m/z 44 from the carboxyl group (Nadeem, Mumtaz, Danish, Rashid, Mukhtar & Irfan, 2020), and this MS cleavage pattern is confirmed in 2.02 min peak (Fig. 1b). RUA with a

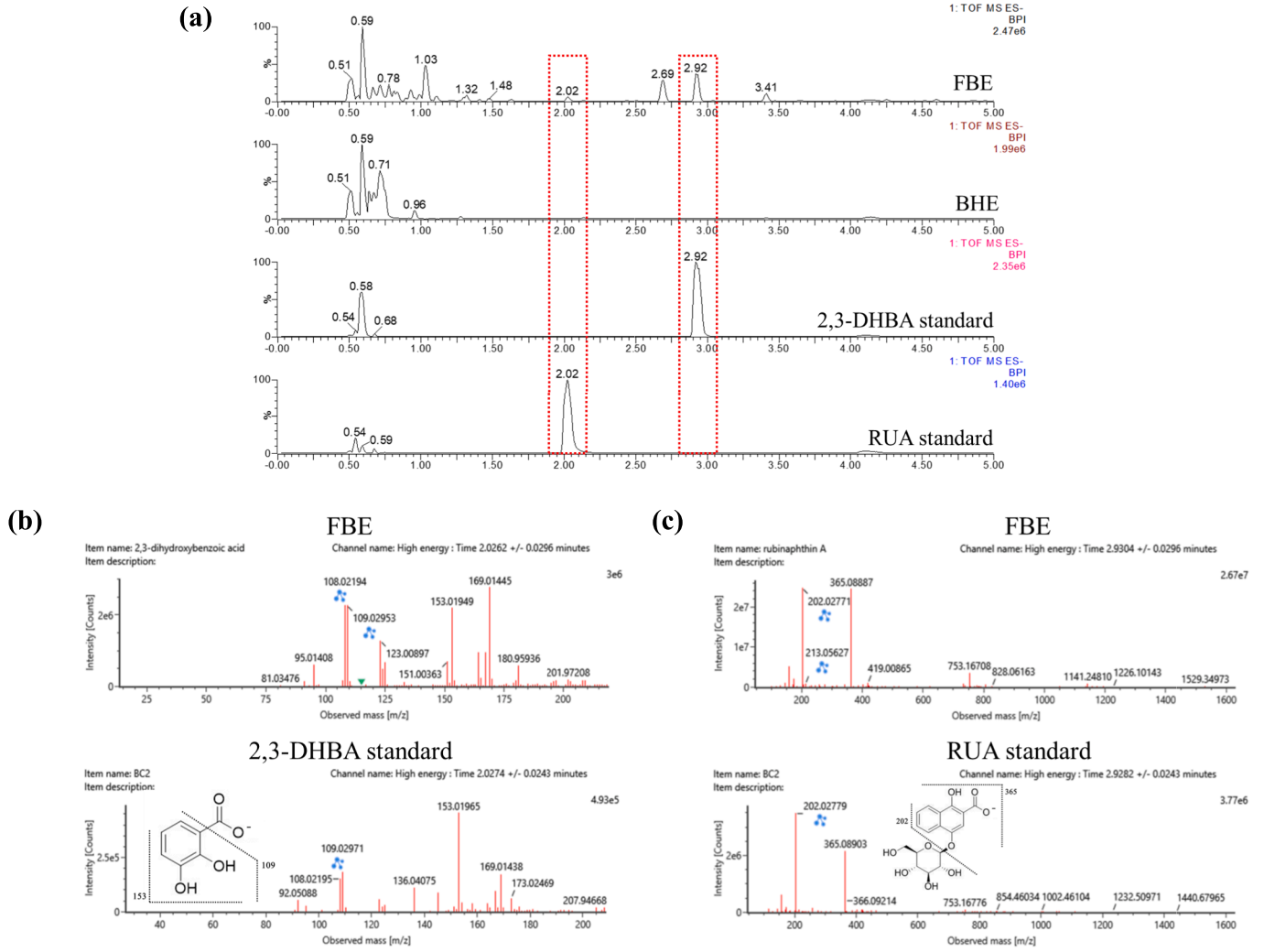

Fig. 1. Total ion current chromatograms (a) and mass spectra (b and c) of fermented *Benincasa hispida* (FBE), 2,3-dihydroxybenzoic acid (2,3-DHBA), and rubinaphthin A (RUA) as determined by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry/mass spectrometry (UPLC-QTOF-MS/MS).

Table 1
Tentative identification of compounds in fermented *Benincasa hispida* extracts (FBE).

RT (min)	Detected ion	Calculated ion	Adduct ion	Mass error (ppm)	Fragment ion	Molecular formula
2.02	153.0195	153.0188	**[M-H]** $^-$	3.3	109.029	$C_7H_6O_4$
2.92	365.0889	365.0873	**[M-H]** $^-$	−2.2	202.027	$C_{17}H_{18}O_9$

pseudo-molecular ion [M-H]$^-$ at *m/z* 365 gets fragmented to 202 [(M-H) − 163]$^-$ due to the loss of *m/z* 163 from the glycosyl residue. Comparison of the MS, PDA spectra, and RTs of standard compounds with that of generated compounds further confirm the identity of the latter ones as 2,3-DHBA and RUA (Fig. 1b and c).

3.2. Chromatographic method validation

3.2.1. Specificity and confirmation of identity

Compounds containing conjugated double bonds, benzene rings, carboxyl groups, hydroxyl groups, and other structures generally exhibit UV absorption (Lin et al., 2021). 2-FA has a structure bonded to a carboxylic acid group and 2-furyl, and the maximum absorption wavelength of furan appears at 250 nm (Shi, Zhang, Zong, Wang & Li, 2019). The carboxyl group in the 2,3-DHBA structure also exhibits an absorption peak in the range of 200–280 nm. Moreover, 2,3-DHBA and RUA, as phenolic compounds, also exhibit a UV absorption peak at 270 nm. Suitable simultaneous determination of marker compounds of BHE and FBE, including 2-FA, 2,3-DHBA, and RUA, was performed using the HPLC-PDA method. The identity of the analyte confirmed from the UV spectrum of the standard spiked with sample matrix is shown in Fig. 2(a-c). The HPLC-PDA system generated well-separated peaks of 2-FA, 2,3-DHBA, and RUA matching the RTs and UV spectrum, and degree of separation with an Rs value > 2 are shown in Fig. 2 (d and e). The method specificity was determined by comparing the RTs and PDA spectra of standards as follows: 2-FA (λ_{max} = 252 nm; RT = 8.1 min), 2,3-DHBA (λ_{max} = 206, 247, and 316 nm; RT = 13.7 min), and RUA (λ_{max} = 214, 256, and 353 nm; RT = 16.9 min).

3.2.2. Linearity, LOD, and LOQ

Validation of indicator component analysis methods is a process of establishing standard analysis methods performed on the developed

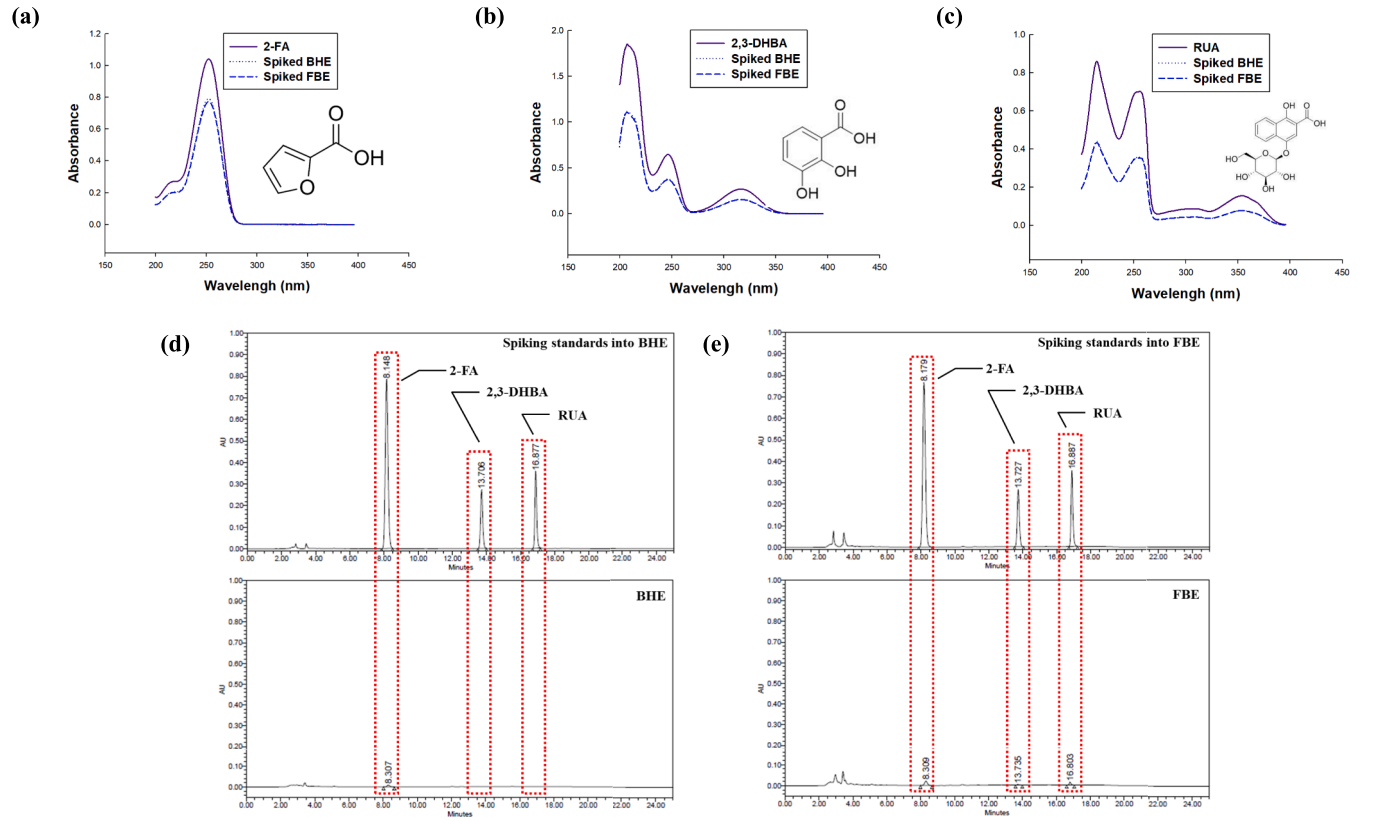

Fig. 2. Photodiode array (PDA) spectra (a-c) and chromatograms (d and e) of non-fermented (BHE) and fermented *Benincasa hispida* extracts (FBE) spiked with 2-furoic acid (2-FA), 2,3-dihydroxybenzoic acid (2,3-DHBA), and rubinaphthin A (RUA) as determined by high-performance liquid chromatography (HPLC)-PDA detection.

analysis method or applied analysis matrix, and the application of standard analysis methods is essential in the standardization of functional raw materials and processes. The HPLC-PDA analytical method for the quantification of three marker compounds (2-FA, 2,3-DHBA, and RUA) in BHE and FBE was validated according to the following procedures: linearity, LOD, LOQ, precision, and accuracy. Linearity of a analysis method refers to the ability to obtain linear measurement values within a certain range in proportion to the amount (or concentration) of the analyte in the sample. The linearity of the calibration curves was determined using a seven-point concentration (1.56–100 μg/mL) of the standard mixture. As shown in Table 2, the coefficient of determination (R^2) for all the marker compounds was 0.9993. LOD refers to the minimum detectable amount of an analyte present in a sample, and LOQ refers to the minimum amount of an analyte in a sample that can be expressed as a quantitative value with appropriate precision and accuracy. These are validation parameters for quantitative tests of samples containing trace amounts of the analyte, especially those used to determine impurities and decomposition products. The LOD and LOQ values were determined based on calibration standard curves and ranged from 0.13 to 0.36 μg/mL and 0.41 to 1.09 μg/mL, respectively.

3.2.3. *Precision and accuracy*

Precision refers to the proximity (degree of dispersion) between values collected from a sample multiple times and analyzed. Accuracy refers to the degree to which an analysis value is close to a known true value or standard value. Precision and accuracy were determined by repeatability (three times repeated in a day) and intermediate precision (three times repeated in three different days) based on three spiked concentrations of the marker compound. Precision was expressed in terms of RSD, and accuracy was assessed as the percent recovery of the analyte concentration measured (Table 3). The intraday and interday precision ranges in BHE were 0.04–0.31 % for 2-FA, 0.20–0.89 % for 2,3-DHBA, 0.12–1.05 % for RUA. In FBE, the average RSDs were 0.11–0.64 % for 2-FA, 0.11–0.89 % for 2,3-DHBA, 0.19–1.42 % for RUA, respectively. The average recoveries in BHE were 87.84–102.41 % for 2-FA, 89.02–102.51 % for 2,3-DHBA, 86.82–100.94 %, respectively. In FBE, the average recoveries were 95.60–100.37 % for 2-FA, 95.96–100.45 % for 2,3-DHBA, 95.85–100.62 % for RUA. Method validation is the essential process for ensuring that the analytical procedure employed for a particular problem is fit for its intended purpose. In this study, standardization is needed to control quality from *B. hispida* raw materials to fermentation process and FBE product. In particular, the verification of analytical methods in complex matrices such as the production of many

Table 2
Coefficient of determination of the calibration curves, limit of detection (LOD), and limit of quantitation (LOQ) of bioactive compounds analysis.

Analytes	Range (μg/mL)	Slope	Intercept	Coefficient of Determination (R^2)	LOD [1] (μg/mL)	LOQ [2] (μg/mL)
2-FA [3]	1.56–100	171789.69	58850.05	0.9993	0.15	0.45
2,3-DHBA [4]	1.56–100	47027.27	6799.66	0.9993	0.13	0.41
RUA [5]	1.56–100	53765.22	19447.63	0.9993	0.36	1.09

[1] LOD is the limit of detection. [2] LOQ is the limit of quantification. [3] 2-FA is the 2-furoic acid. [4] 2,3-DHBA is the 2,3-dihydroxybenzoic acid. [5] RUA is the rubinaphthin A.

Table 3
Recoveries of bioactive compounds (three different concentrations) in non-fermented (BHE) and fermented *Benincasa hispida* extracts (FBE).

Matrix	Analytes		Concentration (μg/mL)	Mean ± SD (μg/mL)	RSD [1] (%)	Recovery (%)
BHE	2-FA [2]	Intraday	12.5	10.98 ± 0.01	0.05	87.84
			25	22.31 ± 0.02	0.11	89.25
			50	51.12 ± 0.04	0.07	102.23
		Interday	12.5	11.16 ± 0.00	0.04	89.29
			25	22.47 ± 0.02	0.07	89.88
			50	51.21 ± 0.16	0.31	102.41
	2,3-DHBA[3]	Intraday	12.5	11.17 ± 0.02	0.20	89.36
			25	22.26 ± 0.20	0.89	89.02
			50	51.25 ± 0.10	0.20	102.51
		Interday	12.5	11.27 ± 0.10	0.85	90.12
			25	22.52 ± 0.20	0.89	90.07
			50	51.23 ± 0.34	0.67	102.46
	RUA[4]	Intraday	12.5	10.85 ± 0.01	0.12	86.82
			25	22.08 ± 0.03	0.14	88.34
			50	50.47 ± 0.08	0.16	100.94
		Interday	12.5	11.03 ± 0.04	0.41	88.22
			25	22.15 ± 0.17	0.75	88.59
			50	50.47 ± 0.53	1.05	100.94
FBE	2-FA	Intraday	12.5	11.95 ± 0.02	0.13	95.60
			25	24.33 ± 0.12	0.47	97.33
			50	50.20 ± 0.06	0.11	100.37
		Interday	12.5	12.14 ± 0.06	0.52	97.13
			25	24.43 ± 0.16	0.64	97.73
			50	50.14 ± 0.21	0.42	100.28
	2,3-DHBA	Intraday	12.5	11.99 ± 0.01	0.11	95.96
			25	24.33 ± 0.11	0.43	97.31
			50	50.22 ± 0.06	0.11	100.45
		Interday	12.5	12.17 ± 0.02	0.19	97.33
			25	24.45 ± 0.04	0.16	97.80
			50	50.19 ± 0.45	0.89	100.38
	RUA	Intraday	12.5	11.98 ± 0.02	0.19	95.85
			25	24.37 ± 0.13	0.54	97.47
			50	50.31 ± 0.12	0.24	100.62
		Interday	12.5	12.19 ± 0.10	0.86	97.55
			25	24.45 ± 0.35	1.42	97.80
			50	50.26 ± 0.36	0.72	100.52

[1] RSD is the relative standard deviation. [2] 2-FA is the 2-furoic acid. [3] 2,3-DHBA is the 2,3-dihydroxybenzoic acid. [4] RUA is the rubinaphthin A.

metabolites during the fermentation process becomes more important. (Cortese, Gigliobianco, Magnoni, Censi & Di Martino, 2020). The method validation guidelines presented by the ICH and US FDA allow accuracy and precision levels within the range of 80–120 % and less than 5 %, respectively (FDA, 2020; FDA, 2021). Therefore, the HPLC–PDA method for three marker compounds (2-FA, 2,3-DHBA, and RUA) showed excellent precision and accuracy at all concentrations in the BHE and FBE matrices.

3.3. Quantification of targeted marker compounds by the developed method

Fermentation induced an increase in the content and production of marker compounds. The contents of marker compounds analyzed in BHE and FBE quantified using the developed method are shown in Table 4. The HPLC-PDA-based method shows that BHE contains only 273.00 ± 0.60 μg/mL of 2-FA, whereas FBE contains 672.24 ± 6.65 μg/mL of 2-FA, 241.32 ± 9.25 μg/mL of 2,3-DHBA, and 732.30 ± 6.66 μg/mL of RUA (Table 4). In the HPLC-PDA-based method, only 2-FA was identified in BHE, whereas fermentation induced an increase in the 2-FA content and production of 2,3-DHBA and RUA within the acceptable range of LOD and LOQ. Some metabolites, such as furfural, furfuryl alcohol, and 3-methyl-1H-pyrrole, are typically linked to the degradation of sugars and/or amino acids by *B. subtilis*. In particular, the carbonyl group in furfural can be reduced or oxidized to produce furfuryl alcohol, 2-FA, levulinic acid, or furans (Park, Lee & Kim, 2022). Also, Chen et al. (2019) reported that the amino acid conjugate of 2,3-DHBA constitutes a major class of siderophores in *B. subtilis* and produces 2,3-DHBA under iron deficiency. 2,3-DHBA is also an intermediate in the catabolism of L-tryptophan. In the progression of this pathway, anthranilate undergoes deamination facilitated by the enzyme anthranilate hydroxylase, resulting in the formation of 2,3-DHBA (Huccetogullari, Luo & Lee, 2019). Additionally, Kumar et al. (2023) reported that fermentation using *B. subtilis* can induce biosynthesis and the production of RUA as a secondary metabolite. RUA is a form of the sugar D-glucose linked to-1,4-dihydroxy-2-naphthoic acid through a glycosidic bond by the action of a glycosyltransferase enzyme. Therefore, this study suggests the possibility of using the products increased and produced during the fermentation process as marker compounds (2-FA, 2,3-DHBA, and RUA) in the development of functional foods of FBE. However, as the increase and production of selected marker compounds are presented as explanations and possibilities, further studies of their metabolic pathways are needed. Additionally, various physiological functions of marker compounds of FBE have been reported. 2-FA has been reported to have various physiological activities, such as cholesterol reduction, blood sugar control, and body fat reduction (Liu et al., 2018). 2,3-DHBA, a microbial flavonoid metabolite, has been reported to have anti-inflammatory, anti-diabetic, and antioxidant effects

Table 4
High-performance liquid chromatography-photodiode array (HPLC-PDA) determination of bioactive compounds in non-fermented (BHE) and fermented *Benincasa hispida* extracts (FBE).

Sample	Analytes	Mean ± SD (μg/g)	RSD (%)
BHE	2-FA [1]	273.00 ± 0.60	0.22
	2,3-DHBA [2]	N.D. [4]	–
	RUA [3]	N.D	–
FBE	2-FA	672.24 ± 6.65	0.99
	2,3-DHBA	241.32 ± 9.25	3.83
	RUA	732.30 ± 6.66	0.91

[1] 2-FA is the 2-furoic acid. [2] 2,3-DHBA is the 2,3-dihydroxybenzoic acid. [3] RUA is the rubinaphthin A. [4] ND is the not detected.

(Cilleros, López-Oliva, Martín & Ramos, 2020). Therefore, future studies should analyze compounds of FBE that play an important role in enhancing bioactive effects.

4. Conclusion

The purpose of this study was to develop marker compounds and verify analytical methods for the standardization of BHE and FBE, which have been used for nutritional and medicinal purposes in oriental medicine, as functional foods. Many studies on *B. hispida* fruit have focused only on functionality and efficacy. There has been no research on identifying marker compounds considering specificity (existence or differential content variation) and representativeness (involved in function). Therefore, in this study, marker compounds that are increased or produced during the fermentation process were identified based on UPLC-QTOF-MS/MS, and the identified marker compounds (2-FA, 2,3-DHBA and RUA) suggested the possibility of using them as standardization indicators in fermentation processes. In addition, to evaluate the ease and usefulness of marker compounds (analysis possible through generalized equipment), analysis method development, validation, and content analysis were performed based on HPLC-PDA for the three marker compounds. From the validation result, the specificity, linearity, LOD, LOQ, precision, and accuracy of the analytical method are found to be excellent. It is believed that the process will be used for standardization in the development of functional foods with FBE. However, further studies are needed to identify the metabolic production pathway through *B. subtilis* fermentation and the active compounds of FBE for physiological functions.

CRediT authorship contribution statement

Sun-Il Choi: Writing – original draft, Visualization, Project administration. **Xiao Men:** Software, Methodology, Investigation. **Geon Oh:** Validation, Software, Formal analysis. **Ji-Hyun Im:** Supervision, Funding acquisition, Conceptualization. **Ye-Eun Choi:** Supervision, Funding acquisition, Conceptualization. **Jung-Mo Yang:** Validation, Resources. **Ju-Hyun Cho:** Supervision, Funding acquisition, Conceptualization. **Ok-Hwan Lee:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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