Chromatographic Evaluation and Characterization of Components of Gentian Root Extract Used as Food Additives

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Gentian root extract is used as a bitter food additive in Japan. We investigated the constituents of this extract to acquire the chemical data needed for standardized specifications. Fourteen known compounds were isolated in addition to a mixture of gentisin and isogentisin: anoninic acid, 2-methoxyanoninic acid, furan-2-carboxylic acid, 5-hydroxymethyl-2-furfural, 2,3-dihydroxybenzoic acid, isovitexin, gentiopicroside, loganic acid, sweroside, vanillic acid, gentisin 7-O-primeveroside, isogentisin 3-O-primeveroside, 6′-O-gluco-sylgentiopicroside, and swertiajaponoside D. Moreover, a new compound, loganic acid 7-(2′-hydroxy-3′-O-β-D-glucopyranosyl)benzoate (1), was also isolated. HPLC was used to analyze gentiopicroside and amarogentin, defined as the main constituents of gentian root extract in the List of Existing Food Additives in Japan.

Key words gentian root extract; Gentiana lutea; food additive; bittering agent; iridoid

Most existing food additives, which are officially registered based on the Food Sanitation Act in Japan, are natural extracts containing various ingredients. The characteristic of food additive ingredients are not always property defined owing to the poor analysis of ingredients in the raw material. In this study, we investigate the constituents of some food additives to acquire the necessary chemical data for standardized specifications. The chemical constituents of gentian root extract, a bitter food additive registered in the List of Existing Food Additives in Japan, were evaluated by HPLC in this study.

Gentiana lutea (Gentianaceae) is an herbaceous perennial plant commonly found in the mountains of Europe and western Asia. The root and rhizome of G. lutea constitute the official drug in the Japanese Pharmacopoeia.3 Previously, phytochemical studies of this drug revealed the presence of bitter secoiridoid glycosides, including gentiopicroside, loganic acid, sweroside, vanillic acid, gentisin 7-O-primeveroside, isogentisin 3-O-primeveroside, 6′-O-glucosylgentiopicroside, and swertiajaponoside D. Moreover, a new compound, loganic acid 7-(2′-hydroxy-3′-O-β-D-glucopyranosyl)benzoate (1), was obtained as a brown amorphous powder, together with the respective n-hexane, EtOAc, n-BuOH, and water extracts. The EtOAc, n-BuOH, and water extracts, which contained aromatic compounds as assessed by HPLC, were separately chromatographed using YMC gel ODS-AQ, Sephadex LH-20, and/or Chromatorex ODS with methanol (MeOH) [or ethanol (EtOH)]–H2O in stepwise gradient mode. The fractions showing similar HPLC patterns were combined and further purified by column chromatography, to afford loganic acid 7-(2′-hydroxy-3′-O-β-D-glucopyranosyl)benzoate (1), together with 5-hydroxymethyl-2-furfural (2),2 furan-2-carboxylic acid (3),3 2,3-dihydroxybenzoic acid (4),4 gentiopicroside (5),5 isovitexin (6),5 gentisin 7-O-primeveroside (7),5 isogentisin 3-O-primeveroside (8),6 a mixture of gentisin (9) and isogentisin (10),6 vanillic acid (11),7 loganic acid (12),5 sweroside (13),5 6′-O-gluco-sylgentiopicroside (14),5 and swertiajaponoside D (15).8 The n-hexane extract was dissolved in acetone and subjected to preparative TLC to give anonic acid (16) and 2-methoxyanofinic acid (17).9 The known compounds were identified by direct comparison with authentic specimens or by spectral comparison with data reported in the literature (Fig. 1).

Compound 1 was obtained as a brown amorphous powder, and its molecular formula was assigned as C30H33O18 by high resolution-electrospray ionization (HR-ESI)-MS (m/z 673.1970 [M-H]-).10 Caled for C30H33O18: 673.1985 and 13C-NMR (29.13C signals). The UV spectrum of 1 showed absorption maxima at 216, 240, and 312 nm. The presence of the loganic acid moiety was indicated in the 1H-NMR spectrum by an olefin proton (1H, δ 7.39), five methine protons (each 1H, δ 2.16, 2.27, 5.47, 3.15–3.53, 5.30), methylene protons (each 1H, δ 1.89, 2.45), a methyl proton (3H, δ 1.14), an anomic proton (1H, δ 4.91), and aliphatic protons corresponding to the sugar...
unit, in addition to 16 carbon resonances assignable to C-1–11 and glucose C-1–6. Signals corresponding to a tri-substituted benzene (2,3-dihydroxybenzoic acid) and another sugar unit were also observed. As compared to the $^1$H-NMR data of 1 and loganic acid, a marked downfield shift ($\delta$ 4.04→5.47) of H-7 was observed by $^1$H–$^1$H correlation spectroscopy (COSY). The linking position of each unit was confirmed by cross-peaks among the sugar H-1′ ($\delta$ 4.68) and tri-substituted benzene C-3′ ($\delta$ 147.3), loganic acid H-7 ($\delta$ 5.47) and ester carbonyl C-7′ ($\delta$ 170.9), and tri-substituted benzene H-6′ ($\delta$ 7.56) and C-7′ via heteronuclear multiple bond correlation (HMBC) (Fig. 2). Additionally, 1 was chemically substantiated by acid hydrolysis followed by HPLC analysis, which confirmed the production of loganic acid. The second sugar unit obtained upon the acid hydrolysis of 1 was confirmed to be D-glucose, by comparing its HPLC data with that of its authentic thiazolidine derivative that was prepared in a separate experiment.
according to a previously reported method. Two \( \beta \)-glycosidic linkages in the glucose cores were evidenced by large coupling constants (each \( J = 7.5 \) Hz). Therefore, the structure of 1 was established as depicted in Figs. 1 and 2.

The HPLC chromatogram of the gentian root extract is shown in Fig. 3. Gentiopicroside (5) was detected as the major component, but amarogentin, which is reported as the other major constituent in the Food Additives list, was not isolated. To ensure quality, we quantitatively analyzed these two compounds. The gentian root extract product (Ms; 1.0 g) was dissolved in MeOH (100 mL; sample solution). The standards [gentiopicroside (5) (Gs) and amarogentin (As)] (0.01 g each) were dissolved in MeOH (100 mL; standard solutions). Aliquots (2 \( \mu \)L) of sample and standard solutions were subjected to analytical HPLC according to the following conditions, and the peak areas, \( A_{ta} \) and \( A_{sa} \) [\( A_{ta} \) and \( A_{sa} \) are peak areas of test solution and standard solution of gentiopicroside (5)] (\( A_{tb} \) and \( A_{sb} \) are those of amarogentin) were determined: content (\%) of gentiopicroside and amarogentin was calculated by \( G_{s} (g) / M_{s} (g) \times A_{ta} / A_{sa} \times 100 \) and \( A_{s} (g) / M_{s} (g) \times A_{tb} / A_{sb} \times 100 \), respectively. The HPLC chromatograms of the gentian root extract prepared using the developed methods via isocratic HPLC are depicted in Fig. 4. The detection limit of gentiopicroside (5) was 0.0013 mg/mL, while that of amarogentin was 0.0008 mg/mL. The amount of gentiopicroside (5) in the extract was 3.7\%, and that of amarogentin was less than the determination limit.

The bitter principles of the gentian root extract were at-
tributed to two secoiridoid glucosides [gentiopicroside (5) and amarogentin].\(^\text{20}\) Since gentiopicroside (5) was detected as the main constituent and amarogentin could not be detected owing to its level being below the detection limit, gentiopicroside (5) was considered the primary contributor to the bitter taste of the gentian root. Previously, amarogentin was reported to be one of the main bitter components of gentian\(^\text{2,6}\); however, its content decreases over the cultivation period. Amarogentin content in materials cultivated for more than 5 years was between 0.2–0.4 mg/g. In addition, in the content of amarogentin in the market products of gentian varies markedly. In contrast, contents of gentiopicroside (5) in fresh raw materials.\(^\text{9}\) Therefore, detection of gentiopicroside (5) and amarogentin are influenced by the status of raw materials. The detection of both or either one of these two ingredients is suitable for evaluation of the quality of gentian root extract as food additives.

**Experimental**

**General** Optical rotations were measured with a JASCO P-1030 digital polarimeter (Tokyo, Japan). UV spectra were recorded on a Shimadzu UVmini-1240 (Kyoto, Japan) and a JASCO V-530 (Tokyo, Japan). ESI-MS and HR-ESI-MS spectra were obtained using a microTOF-Q (Bruker Daltonics, Billerica, MA, U.S.A.) mass spectrometer with acetonitrile as the solvent. \(^1\)H- and \(^13\)C-NMR spectra were recorded on a Bruker AVANCE500 instrument (Bruker BioSpin, Billerica, MA, U.S.A.) (at 500 MHz and 126 MHz, respectively) and chemical shifts are given in ppm relative to those of the internal standard TMS at 0 ppm. 2D NMR spectra were used for each 2D measurement [COSY, heteronuclear single quantum coherence (HSQC), and HMBC].

**Samples and Reagents** Commercial gentian root extract was obtained from the Japan Food Additives Association (JFA) (Tokyo, Japan). The standard compounds (gentiopicroside and amarogentin) used for quantitative analysis were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were of analytical grade.

**Extraction and Isolation** The gentian root extract (40 g) was dissolved in \(\text{H}_2\text{O} (1 \text{~L})\), and then extracted with \(n\)-hexane (3 L), EtOAc (3 L), and \(n\)-BuOH (3 L) to yield \(n\)-hexane (97.2 mg), EtOAc (989.5 mg), \(n\)-BuOH (3.8 g) and water (33.8 g) extracts, respectively. The EtOAc extract (760.1 mg) was chromatographed over YMC gel ODS-AQ with MeOH–\(\text{H}_2\text{O} (0:100\rightarrow10:90\rightarrow20:80\rightarrow30:70\rightarrow40:60\rightarrow100:0)\) in stepwise gradient mode. The fractions showing similar HPLC patterns (Condition 1) were combined and further purified by column chromatography over Sephadex LH-20 with EtOH and/or Chromatorex ODS with aqueous MeOH to afford 5-hydroxymethyl-2-furfural (2) (14.2 mg), furan-2-carboxylic acid (4.3 mg) (3), 2,3-dihydroxybenzoic acid (1.0 mg) (4), gentiopicroside (5) (73.2 mg), isovitexin (6) (4.5 mg), gentisin 7-O-primeveroside (7) (2.6 mg), isogentisin 3-O-primeveroside (8) (19.9 mg), together with mixture of gentisin (9) and isogentisin (10). The \(n\)-BuOH extract (2.0 g) was separated by column chromatography over YMC gel ODS-AQ and Chromatorex ODS with aqueous MeOH to yield gentiopicroside (5) (101.9 mg), loganic acid (12) (51.1 mg), sweroside (13) (9.3 mg), and compound 1 (4.0 mg). The water extract (30 g) was similarly separated by column chromatography over YMC gel ODS-AQ and Chromatorex ODS with aqueous MeOH to yield gentiopicroside (5) (101.9 mg), loganic acid (12) (51.1 mg), sweroside (13) (9.3 mg), and compound 1 (4.0 mg).

### Table 1. \(^1\)H- (500 MHz) and \(^13\)C-NMR (126 MHz) Data of Compound 1 Measured in MeOH-\(d_4\)

| Position | \(\delta_C\) | \(\delta_H\) (J in Hz) |
|----------|-------------|----------------------|
| 1        | 97.4        | 5.30 (d, \(J = 5.5\)) |
| 3        | 151.7       | 7.39 (d, \(J = 1.0\)) |
| 4        | 114.0       | —                    |
| 5        | 33.1        | 3.15–3.53\(^a\)      |
| 6        | 40.5        | 1.89 (m), 2.45 (dd, \(J = 8.0, 8.5\)) |
| 7        | 80.4        | 5.47 (brt, \(J = 5.0\)) |
| 8        | 41.2        | 2.27 (m)             |
| 9        | 47.2        | 2.16 (m)             |
| 10       | 13.8        | 1.14 (3H, d, \(J = 7.5\)) |
| 11       | 171.5       | —                    |
| 1'       | 115.0       | —                    |
| 2'       | 153.0       | —                    |
| 3'       | 147.3       | —                    |
| 4'       | 123.8       | 7.41 (dd, \(J = 1.5, 8.0\)) |
| 5'       | 120.1       | 6.88 (t, \(J = 8.0\)) |
| 6'       | 124.5       | 7.56 (dd, \(J = 1.5, 8.0\)) |
| 7'       | 170.9       | —                    |

**Glucose (Glc)-1**

|   | 100.2 | 4.91 (d, \(J = 7.5\)) |
| 2, 2' | 74.8 | 74.9 | 3.15–3.53\(^a\) |
| 3, 3' | 77.0 | 78.0 | 3.15–3.53\(^a\) |
| 4, 4' | 71.3, 71.7 | 3.15–3.53\(^a\) |
| 5, 5' | 78.3, 78.4 | 3.15–3.53\(^a\) |
| 6, 6' | 62.5, 62.8 | 3.67 (dd, \(J = 5.5, 12\)), 3.69 (dd, \(J = 5.0, 12\)), 3.88 (dd, \(J = 2.0, 12\)), 3.91 (dd, \(J = 2.0, 12\)) |

**Glucose (Glc)-1**

|   | 103.1 | 4.68 (d, \(J = 7.5\)) |

\(^a\) Overlapped signals.
Loganic Acid 7-(2'-Hydroxy-3′-O-β-D-glucopyranosyl)benzoate (1)

A light brown amorphous powder. UV $\lambda_{max}$ (MeOH) nm (log $d$): 216 (4.18), 240 (4.01), 312 (3.47). $[\alpha]_D^{26} -48^\circ$ (c=0.1, MeOH). $^1$H-NMR (500 MHz, MeOH-d$_4$) and $^{13}$C-NMR (126 MHz, MeOH-d$_4$) data are provided in Table 1. HR-ESI-MS $m/z$: 673.1970 ([M−H]−). Calcd for C$_{29}$H$_{38}$O$_{13}$H: 673.1985.

Partial Acid Hydrolysis of Compound 1 A solution of compound 1 (1.0 mg) in H$_2$O (1 mL) and 1 mol/L HCl (0.1 mL) was heated in a boiling water bath for 24 h. After removal of the solvent, the residue was analyzed by HPLC (Condition 1) and loganic acid (12) was detected.

Determination of Sugar Configuration of Compound 1 The sugar units of 1 were determined using a previously described method. Compound 1 (1.0 mg) was hydrolyzed by heating in 0.5 mol HCl (0.2 mL). After neutralization with Amberlite IRA400 followed by evaporation of the solvent, the residue was dissolved in pyridine (0.2 mL) containing L-cysteine methyl ester hydrochloride (1.0 mg) and was heated at 60°C for 1 h. o-Tolyl isothiocyanate (1.0 mg) in pyridine (0.2 mL) was then added to the mixture and heated at 60°C for 1 h. The reaction mixture was directly analyzed by RP-HPLC (Condition 2). The peak coincided with that of the derivative of the authentic sample, β-glucose.

Quantitative Analysis of Gentiotricoside (5) and Amarogentin The gentian root extract product (Ms; 1.0 g) was dissolved in MeOH (100 mL; sample solution). The standards [gentiotricoside (5) (Gs)] and amarogentin (As)] (0.01 g each) were dissolved in MeOH (100 mL; standard solutions). Aliquots (2 µL) of sample and standard solutions were subjected to analytical HPLC (Condition 3 for gentiotricoside (5) and 4 for amarogentin) according to the following conditions, and the peak areas, Ata and Asa, of gentiotricoside (5) (Atb and Asb, of amarogentin) were determined: content (%) of gentiotricoside (5) and amarogentin was calculated by Gs (g)/Ms (g)×Ata/Asa×100 and As (g)/Ms (g)×Atb/Asb×100, respectively.

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Conflict of Interest The authors declare no conflict of interest.