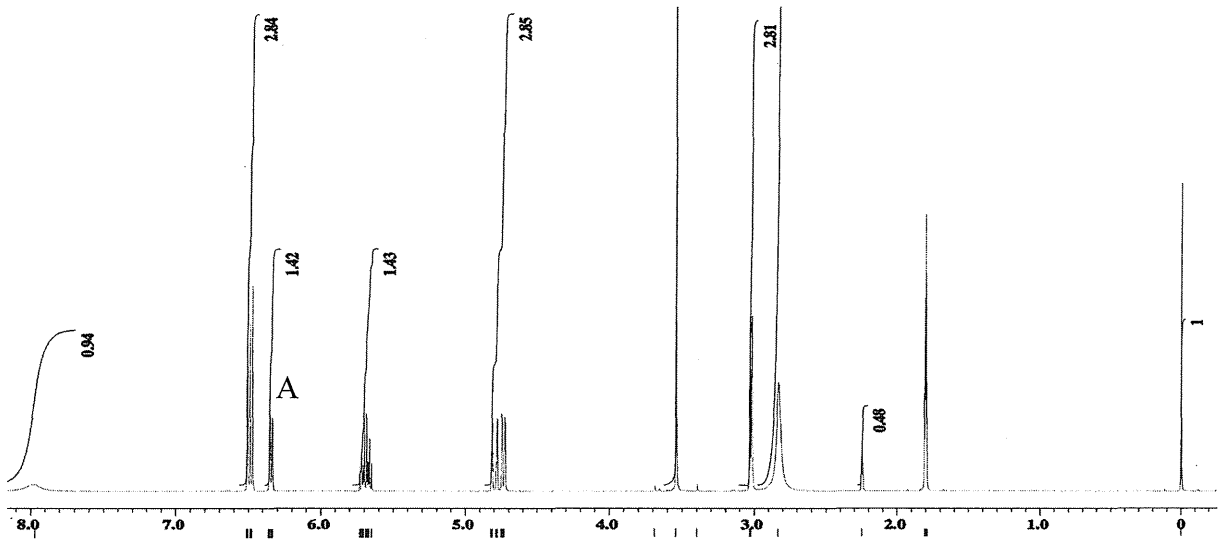

Fig. 7　Eugenol の ^{1}H-NMR スペクトル (aceton-d_6, 500 MHz)

A: eugenol の 6 位 H のシグナル

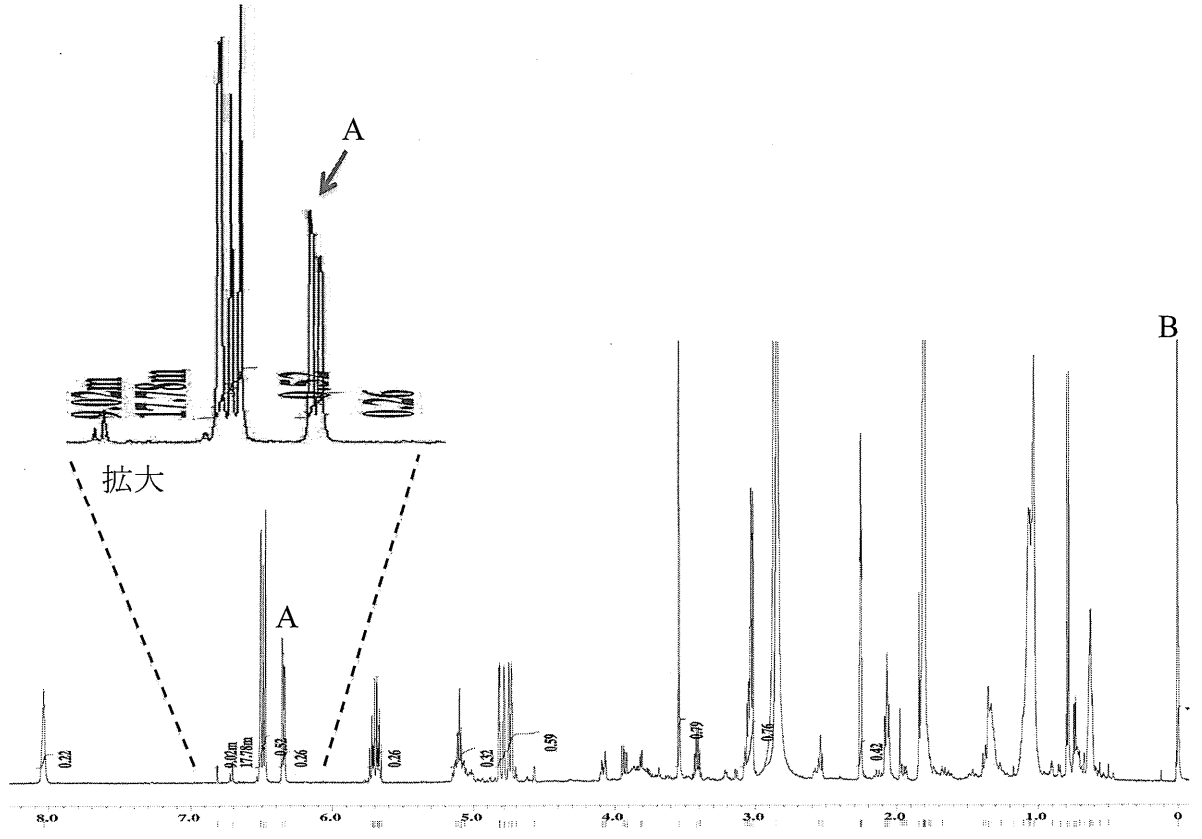

Fig. 8　「クローブ抽出物」の ^{1}H-NMR スペクトル (aceton-d_6, 500 MHz)

A: eugenol の 6 位 H のシグナル、B: 1,4-BTMSB-d_6 のメチル基のシグナル

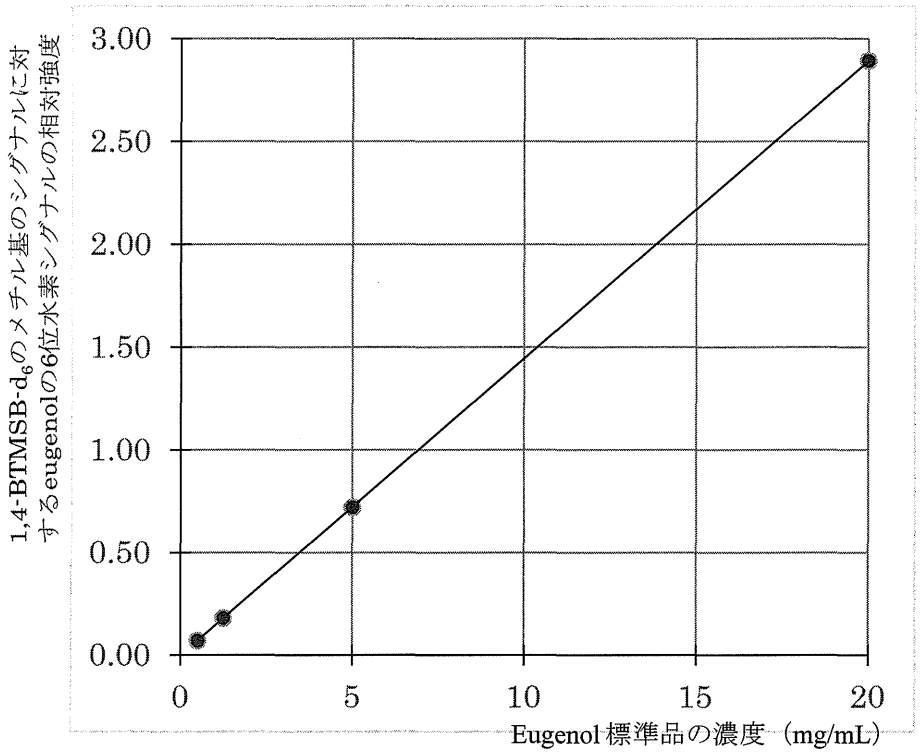

Fig. 9　Eugenol 標準品で作成した検量線　(γ=1.000)

Table 1 ^{1}H-qNMR スペクトルの測定条件

分光計	日本電子　ECA500
観測範囲	−5 ～ 15 ppm
データポイント数	32000
フリップアングル	90°
パルス待ち時間	60 秒
積算回数	8 回
スピン	なし
プローブ温度	25℃

Table 2　^{1}H-qNMR 法から定量された「グルコサミン」中の glucosamne の含有率

	含有率（%）		
	グルコサミン[a)]として		塩酸塩[b)]として
試料	平均±SEM（n=3）		平均
A	81.38	±2.21	
B	82.26	±1.46	
C	81.82	±1.28	
塩酸塩	79.21	±1.71	95.36
塩酸塩粉砕品	81.00	±1.47	97.52

[a)] $C_6H_{13}NO_5$: MW 179 としての算出

[b)] $C_6H_{13}NO_5$・HCl : MW 215.5 としての算出

Table 3　^{1}H-qNMR 法で定量された eugenol の含有率

samples	含有率(%) 平均±SEM（n=5）	表示
eugenol 標準品		
A	92.47　±1.31	99.8%
クローブ抽出物		
B	30.26　±1.19	
C	30.47　±1.55	38%

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Amakura Y, Yoshimura M, Morimoto S, Yoshida T, Tada A, Ito Y, Yamazaki T, Sugimoto N, Akiyama H	Chromatographic evaluation and characterization of components of gentian root extract used as food additives.	*Chem Pharm Bull*	64	78-82	2016
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多田敦子，石附京子，杉本直樹，吉松嘉代，川原信夫，末松孝子，有福和紀，深井俊夫，田村幸吉，大槻崇，田原麻衣子，山﨑壮，穐山浩	既存添加物カンゾウ油性抽出物の成分組成の多変量解析に基づく基原植物種の検討	日本食品衛生学雑誌	56	217-227	2015
多田敦子，杉本直樹，小林義和，濱田ひかり，石附京子，秋山卓美，伊藤裕才，川原信夫，山﨑壮，穐山浩	味認識装置による既存添加物苦味料及び関連苦味化合物の品質評価	日本食品化学学会誌	22	25-31	2015
西﨑雄三，多田敦子，石附京子，伊藤裕才，小野田絢，杉本直樹，穐山浩	モル吸光係数比を利用したジャマイカカッシア抽出物中のクアシン及びネオクアシンの新規定量法の開発	日本食品衛生学雑誌	56	185-193	2015

Note

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: anofinic acid, 2-methoxyanofinic 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*-glucosylgentiopicroside, and swertiajaposide 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,[1] 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 crude drug "Gentianae Radix," which is used as an herbal stomachic worldwide.[2] Gentianae Radix is also listed as an official drug in the Japanese Pharmacopoeia.[3] Previously, phytochemical studies of this drug revealed the presence of bitter secoiridoid glycosides, including gentiopicroside, swertiamarin, and amarogentin,[4–6] xanthones,[7] phenolic acids,[8] flavonoids,[9] and triterpenoids.[10,11]

Notably, water or ethanolic extract of the gentian root and rhizome is used to impart a bitter taste to liqueurs. Although it is known that the bitterness of gentian root extract is attributable to the presence of gentiopicroside and amarogentin, the bittering agent is not well characterized. Here, we describe the isolation and structural characterization of the constituents of gentian root extract and identify a previously unknown iridoid glycoside. Additionally, the phytochemical profile and quantitative analysis of the constituents of this extract are described and discussed in regard to their use as bittering agents. To the best of our knowledge, this is the first report of the analysis of the constituents of gentian root extract as a food additive.

Results and Discussion

Gentian root extract was dissolved in water and partitioned with *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield 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)]–H_2O 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**),[12] furan-2-carboxylic acid (**3**),[13] 2,3-dihydroxybenzoic acid (**4**),[14] gentiopicroside (**5**),[5] isovitexin (**6**),[15] gentisin 7-*O*-primeveroside (**7**),[7] isogentisin 3-*O*-primeveroside (**8**),[16] a mixture of gentisin (**9**) and isogentisin (**10**),[4] vanillic acid (**11**),[17] loganic acid (**12**),[4] sweroside (**13**),[15] 6′-*O*-glucosylgentiopicroside (**14**),[16] and swartiajaposide D (**15**).[18] The *n*-hexane extract was dissolved in acetone and subjected to preparative TLC to give anofinic acid (**16**) and 2-methoxyanofinic acid (**17**),[4] 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 $C_{29}H_{38}O_{18}$ by high resolution-electrospray ionization (HR-ESI)-MS (*m/z* 673.1970 $[M-H]^-$; Calcd for $C_{29}H_{38}O_{18}$-H: 673.1985) and ^{13}C-NMR (29 ^{13}C 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 ^{1}H-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 anomeric proton (1H, δ 4.91), and aliphatic protons corresponding to the sugar

[#] These authors contributed equally to this work.

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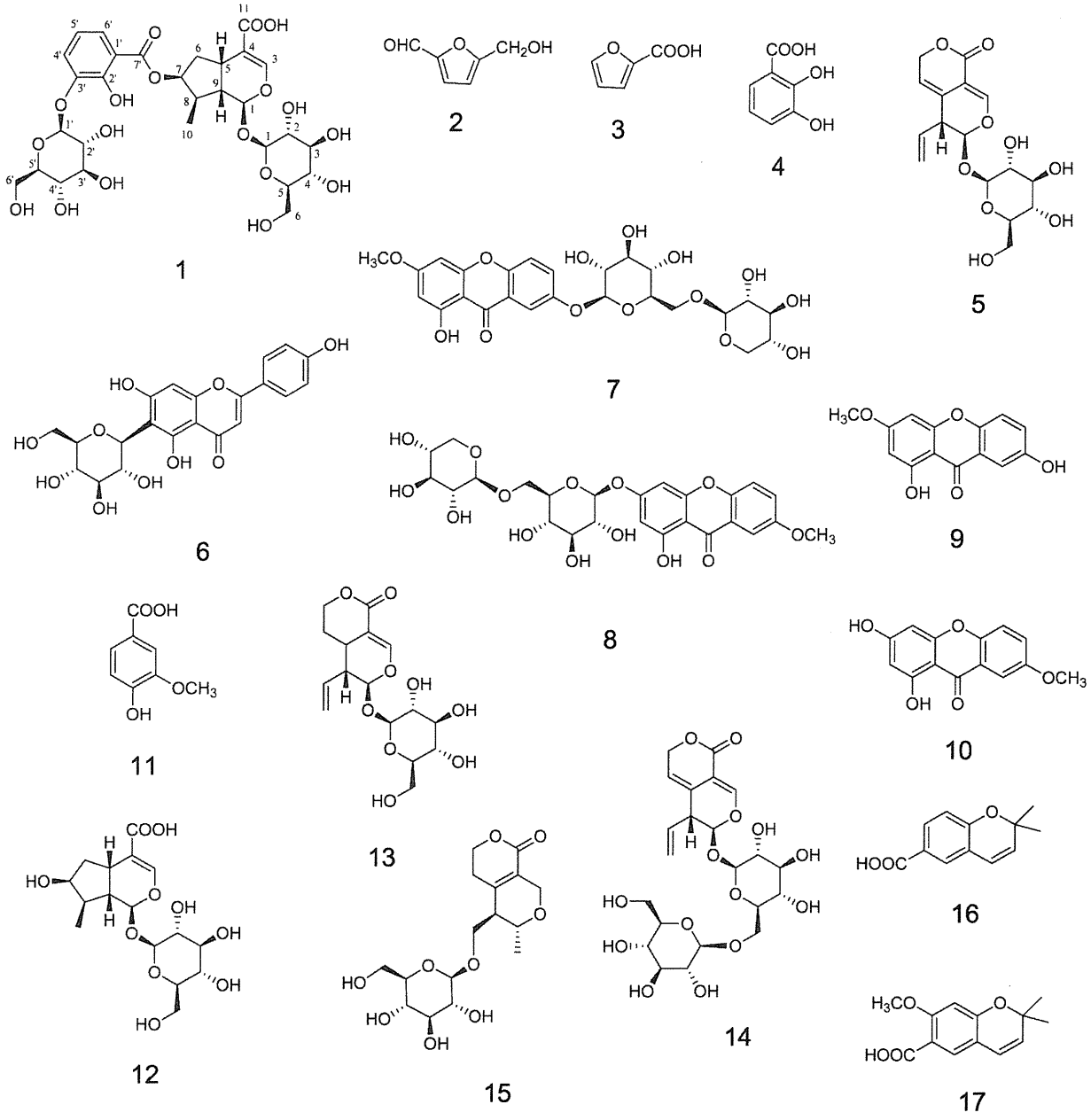

Fig. 1. Structures of Compounds **1**–**17**

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 (δ 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′ (δ 4.68) and tri-substituted benzene C-3′ (δ 147.3), loganic acid H-7 (δ 5.47) and ester carbonyl C-7′ (δ 170.9), and tri-substituted benzene H-6′ (δ 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,

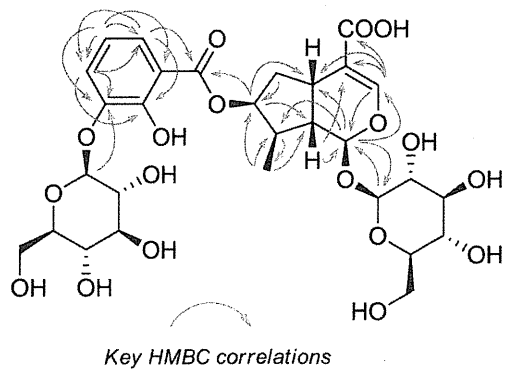

Fig. 2. Selected HMBC Correlations of Compound **1**

according to a previously reported method.[19] Two β-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,[1] 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 μL) of sample and standard solutions were subjected to analytical HPLC according to the following conditions, and the peak areas, *Ata* and *Asa* [*Ata* and *Asa* are peak areas of test solution and standard solution of gentiopicroside (**5**)] (*Atb* and *Asb* are those of amarogentin) were determined: content (%) of gentiopicroside and amarogentin was calculated by Gs (g)/Ms (g)×*Ata*/*Asa*×100 and As (g)/Ms (g)×*Atb*/*Asb*×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-

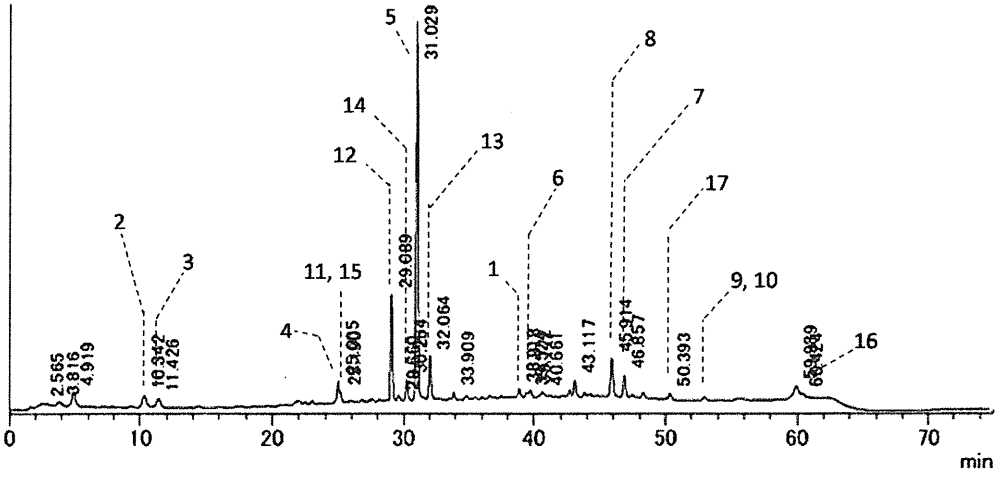

Fig. 3. RP-HPLC Chromatogram of Gentian Root Extract

The number on the chromatogram corresponds to the compound number. Condition is described in Condition 1.

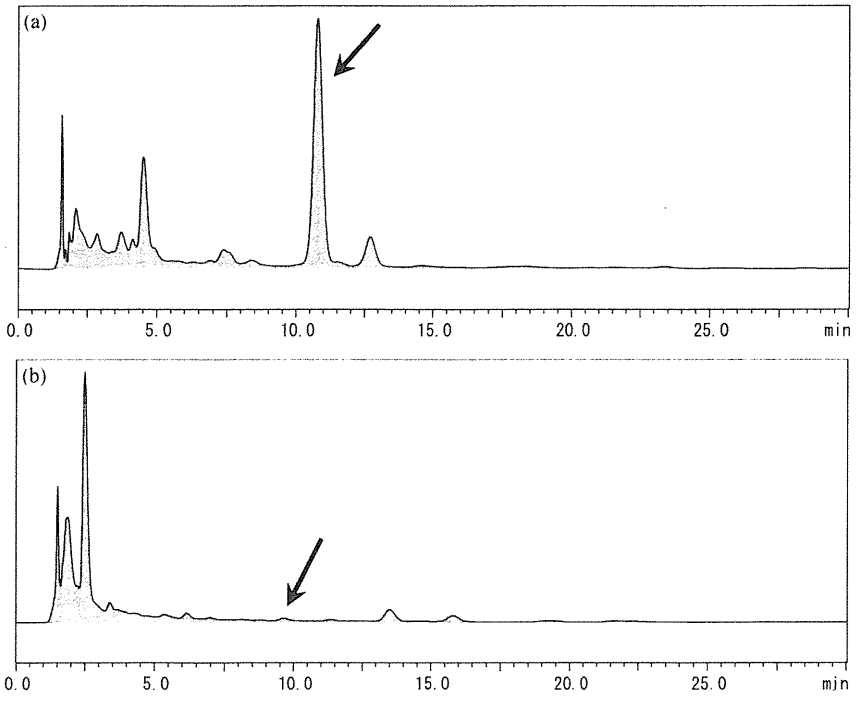

Fig. 4. RP-HPLC Chromatograms of Gentian Root Extract for Quantitative Analysis

(a) Gentiopicroside (Condition 2); (b) Amarogentin (Condition 3).

tributed to two secoiridoid glucosides [gentiopicroside (**5**) and amarogentin].[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[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, the content of gentiopicroside (**5**) in market products was reported to be 30–40 mg/g with low variability.[20] Another study reported the isolation of amarogentin, but not gentiopicroside (**5**), from fresh raw materials.[4] 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 Brucker 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 solvents [acetone-d_6 (δ_H 2.04; δ_C 29.8), MeOH-d_4 (δ_H 3.30; δ_C 49.0)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (AVANCE 500) were used for each 2D measurement [COSY, heteronuclear single quantum coherence (HSQC), and HMBC]. J_{CH} was set at 10 Hz in HMBC. Column chromatography was carried out with MCI-gel CHP-20P (Mitsubishi Chemical Co., Tokyo, Japan), YMC GEL ODS-AQ (YMC Co., Ltd., Kyoto, Japan), Sephadex LH-20 (GE Healthcare, Little Chalfont, U.K.), Chromatorex ODS (Fuji Silysia Chemical Ltd., Aichi, Japan), and Silica Gel 60 (Nacalai Tesque, Kyoto, Japan). Reversed-phase (RP) HPLC conditions were as follows: [Condition 1] column, YMC-pack ODS AQ-3C2 (5 μm, 150×2.0 mm i.d.) (YMC Co., Ltd.); mobile phase, solvent A was 10 mM H_3PO_4–10 mM KH_2PO_4 (1 : 1) and solvent B was MeOH (0–30 min, 0–50% B in A; 30–50 min, 50–60% B in A); injection volume, 3 μL; column temperature, 40°C; flow-rate, 0.25 mL/min; detection, 254 nm. [Condition 2] column, YMC-pack ODS AQ-3C2 (5 μm, 150×2.0 mm i.d.) (YMC Co., Ltd.); mobile phase, 10 mM H_3PO_4–10 mM KH_2PO_4–MeOH (37.5 : 37.5 : 25); column temperature, 40°C; flow-rate, 0.2 mL/min; detection, 250 nm. [Condition 3] column, L-column ODS (5 μm, 150×2.1 mm i.d.) (YMC Co., Ltd.); mobile phase, 5% acetic acid–acetonitrile (95 : 5); column temperature, 40°C; flow-rate, 0.3 mL/min; detection, 254 nm. [Condition 4] column, L-column ODS (5 μm, 150×2.1 mm i.d.) (YMC Co., Ltd.); mobile phase, 5% acetic acid–acetonitrile (82 : 18); column temperature, 40°C; flow-rate, 0.3 mL/min; detection, 254 nm.

Samples and Reagents Commercial gentian root extract was obtained from the Japan Food Additives Association (JAFA) (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 H_2O (1 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–H_2O (0 : 100→10 : 90→20 : 80→30 : 70→40 : 60→100 : 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 vanillic acid (**11**) (4.8 mg), gentisin 7-*O*-primeveroside (**7**) (5.8 mg), isogentisin 3-*O*-primeveroside (**8**) (3.7 mg), loganic acid (**12**) (26.7 mg), gentiopicroside (**5**) (155.8 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

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

Position	δ_C	δ_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.

(**13**) (19.4 mg), 6′-*O*-glucosylgentiopicroside (**14**) (5.4 mg), and swartiajaposide D (**15**) (10.4 mg). The *n*-hexane extract (95 mg) was dissolved in acetone and subjected to preparative TLC [*n*-hexane–EtOAc–HCOOH (4 : 2 : 1)] to yield anofinic acid (**16**) (3.9 mg) and 2-methoxyanofinic acid (**17**) (4.3 mg). These compounds were identified by direct comparison with authentic specimens or by comparison of their spectral data to those reported in the literature. The physical data of compound **1** are given below.

Loganic Acid 7-(2′-Hydroxy-3′-*O*-*β*-D-glucopyranosyl)-benzoate (**1**)

A light brown amorphous powder. UV λ_{max} (MeOH) nm ($\log\varepsilon$): 216 (4.18), 240 (4.01), 312 (3.47). $[\alpha]_D^{26}$ −48° (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 $C_{29}H_{38}O_{18}$-H: 673.1985).

Partial Acid Hydrolysis of Compound 1 A solution of compound **1** (1.0 mg) in H_2O (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.[19] Compound **1** (1.0 mg) was hydrolyzed by heating in 0.5 M 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 isothyocyanate (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, D-glucose.

Quantitative Analysis of Gentiopicroside (5) and Amarogentin 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 μL) of sample and standard solutions were subjected to analytical HPLC (Condition 3 for gentiopicroside (**5**) and 4 for amarogentin) according to the following conditions, and the peak areas, *Ata* and *Asa*, of gentiopicroside (**5**) (*Atb* and *Asb*, of amarogentin) were determined: content (%) of gentiopicroside (**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.

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Chromatographic evaluation of the components of grape skin extract used as food additives

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Abstract

Grape skin extract, a food manufacturing agent registered in the List of Existing Food Additives in Japan, was evaluated by high performance liquid chromatographic (HPLC) method. Chemical constituents of these extracts were separated by repeated column chromatography, and 12 compounds were isolated and characterized as tryptamine, syringic acid, vanillic acid, ethyl gallate, (+)-catechin, (−)-epicatechin, luteoliflavan, quercetin, quercetin 3-*O*-glucuronide, myricetin 3-*O*-glucoside, procyanidin B-1, and procyanidin B-2 by spectroscopic methods. The presence of malvidin 3-*O*-glucoside as the major anthocyanin was confirmed by HPLC. A broad peak forming a swollen base line was attributed to a B type of proanthocyanidins, *i.e.*, a condensed tannin oligomer, the number and weight averaged molecular weights of which were estimated, by gel permeation chromatography (GPC), to be 5999.6 and 21287.7, respectively. The proanthocyanidin content in commercial grape skin extract products was determined by colorimetric analysis with vanillin-sulfuric acid to be around 60% (catechin equivalent value).

Keywords : grape skin extract, HPLC, anthocyanin, proanthocyanidin, tannin

I　Introduction

Grapes (*Vitis* spp.) (Vitaceae) are a familiar fruit consumed globally. They are eaten raw and used to produce wines and juices. In previous phytochemical studies, their skins were reported to be composed of polyphenols such as the anthocyanin glucosides, *viz.* delphinidin, cyanidin, petunidin, peonidin, and malvidin, as well as gallic acid, (+)-catechin, (−)-epicatechin, quercetin, rutin, resveratrol, viniferin, and condensed tannins[1-6]. Other components include the polysaccharides[7], 1-triacontanol, β-sitosterol, and oleanolic acid[3]. In the List of Existing Food Additives in Japan[8], grape skin extract is defined as "an additive obtained from the fruit skin of American grapes or grapes, having the polyphenol as its main constituent." Concerning the origin, manufacturing method, and essential qualities of these additives, the official list states: "This additive is prepared from the pressed lees of the skin of Koshu, Chardonnay, or Riesling grape and all *Vitis labrusca* or *V. vinifera* varieties, by extraction with ethanol at lukewarm or room temperature".

Most existing food additives, which are officially registered under the Food Sanitation Act, are natural extracts containing various ingredients. However, the characteristic and effective components of the existing food additives are not always properly defined due to poor characterization of the ingredients in the respective raw material. Although the polyphenols from whole grapes have been exhaustively investigated, there is little detailed information on the chemical constituents of grape skin extract product. To ensure food safety, analysis and characterization of individual polyphenolic constituents, and establishment of analytical standards for developing national specifications is required.

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In the present study, we investigated the constituents of grape skin extract to accumulate the necessary chemical data for the development of standardized specifications. To the best of our knowledge, this is the first report of the analysis of the constituents of grape skin extract as a food additive.

Ⅱ Materials and Methods

1. Samples and reagents

Commercial grape skin extracts were obtained through the Japan Food Additives Association (JAFA) (Tokyo, Japan). The standard compounds used for identification were tryptamine, quercetin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), vanillic acid (Tokyo Kasei, Tokyo, Japan), (+)-catechin, (−)-epicatechin (Nagara Science, Gifu, Japan), quercetin 3-*O*-glucuronide, myricetin 3-*O*-glucoside (isolated from *Eucalyptus globulus*), procyanidin B-1, procyanidin B-2, malvidin 3-*O*-glucoside, delphenidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and peonidin 3-*O*-glucoside (Funakoshi, Tokyo, Japan). All other chemicals were of analytical reagent grade. Column chromatography was conducted using a Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan), MCI GEL CHP20P (75–150 μm) (Mitsubishi Chemical), YMC GEL ODS-AQ (AQ12S50) (YMC, Kyoto, Japan), and Develosil Spherical Porous Silica Lop ODS (Nomura Chemical Co. Ltd., Aichi, Japan).

2. Instrumentations

UV spectra were recorded on a Shimadzu UVmini-1240 (Kyoto, Japan). Electrospray ionization (ESI)-MS and high-resolution (HR) ESI-MS spectra were obtained using a micrOTOF-Q (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with acetonitrile as the solvent. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 500 instrument (Bruker BioSpin, Billerica, MA, USA) (500 MHz for ^{1}H and 126 MHz for ^{13}C) and chemical shifts are given in ppm values relative to that of the solvent [methanol-d_4 (δ_H 3.30; δ_C 49.0)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (AVANCE 500) were used for each 2D measurement (COSY, HSQC, and HMBC). J_{CH} was set at 10 Hz for HMBC.

HPLC analysis was carried out using a Shimadzu Prominence system (Shimadzu, Kyoto, Japan). Reversed-phase (RP) HPLC conditions were as follows: [Condition 1] column, Cosmosil 5C_{18}-II (5 μm, 150 × 2.1 mm i.d.) (Nacali Tesque, Inc., Kyoto, Japan); mobile phase, solvent A was water (including 0.1% formic acid) and solvent B was acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 85–90% B in A; 50–55 min, 90–100% B in A; 55–60 min, 100% B in A); injection volume, 2 μL; column temperature, 40 °C; flow-rate, 0.3 mL/min; detection, 200–400 nm. [Condition 2] column, L-column ODS (5 μm, 150 × 2.1 mm i.d.) (Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, solvent A was water (including 5% acetic acid) and solvent B was acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 85–90% B in A; 50–55 min, 90–100% B in A; 55–60 min, 100% B in A); injection volume, 2 μL; column temperature, 40 °C; flow-rate, 0.3 mL/min; detection, 520 nm.

3. Extraction and isolation

The grape skin extract (212.1 g) was suspended in water (3 L), and then the water-soluble and water-insoluble fractions were separated by centrifugation (3,000 rpm, 5 min). The water-soluble fraction was chromatographed over Diaion HP-20 with methanol-H_2O (0:100 → 10:90 → 20:80 → 30:70 → 40:60 → 50:50 → 100:0 v/v) by a stepwise gradient mode. The H_2O eluate was extracted with *n*-hexane (1 L), ethyl acetate (3 L), and *n*-butanol (3 L) to give *n*-hexane (2.4 mg), ethyl acetate (10.3 g), *n*-butanol (20.6 g), and water (53.7 g) extracts, respectively.

The ethyl acetate extract (1 g) was chromatographed over YMC gel ODS-AQ with methanol-H_2O (0:100 → 10:90 → 20:80 → 30:70 → 40:60 → 100:0 v/v) by a stepwise gradient mode. The fractions showing similar HPLC patterns (condition 1) were combined and further purified by column chromatography over MCI GEL CHP20P and Develosil Spherical Porous Silica Lop ODS with aqueous methanol to afford luteoliflavan (2)[9] (6.5 mg), procyanidin B-1 (3) (32.5 mg), (+)-catechin (4)[10, 11] (33.6 mg), procyanidin B-2 (6)[12] (35.2 mg), (−)-epicatechin (8)[13] (10.2 mg), and myricetin 3-*O*-glucoside (10)[14] (4.8 mg). The *n*-butanol extract (1.0 g) was separated by column chromatography over YMC gel ODS-AQ with aqueous methanol to give quercetin 3-*O*-glucuronide (11)[15] (15.6 mg). Diaion HP-20 20% methanol eluate (1.0 g) was separated by column chromatography over YMC gel ODS-AQ with aqueous methanol to give tryptamine (1) (2.0 mg), vanillic acid (5) (8.6 mg), syringic acid (7)[16] (10.2 mg), and ethyl gallate (9)[17] (25.3 mg). Similarly quercetin (12)[18] (3.5 mg) was isolated from the methanol eluate (1.0 g). These compounds were identified by direct comparison with authentic specimens (^{1}H-NMR, ESI-MS, and HPLC data) or by comparison of their spectral data to those reported in the literature. On the other hand, water extracts (1.0 g) were chromatographed over MCI GEL CHP20P to give a fraction of condensed tannin oligomers (35 mg) showing only the broad peak and not the other sharp peaks.

Tryptamine (1): off-white amorpous powder. ^{1}H-NMR (CD_3OD) δ 7.55 (1H, d, J = 8 Hz, H-4), 7.36 (1H, d, J = 8 Hz, H-7), 7.13 (1H, s, H-2), 7.11 (1H, brt, J = 8 Hz, H-6), 7.03 (1H, brt, J = 8 Hz, H-5), 3.15, 3.06 (each 2H, t, J = 7 Hz,

-CH_2CH_2-). ESI-MS *m/z* 161 $[M + H]^+$.

Luteoliflavan (2): pale yellow amorphous powder. ^{1}H-NMR (CD_3OD) δ 6.66 (1H, d, *J* = 2 Hz, H-2′), 6.65 (1H, d, *J* = 8 Hz, H-5′), 6.53 (1H, dd, *J* = 2, 8 Hz, H-6′), 5.87 (2H, s, H-6, 8), 3.95 (1H, m, H-2), 2.85 (1H, dd, *J* = 4.5, 14 Hz, H-4), 2.65, 2.64 (each 1H, m, H-3, 4), 2.50 (1H, dd, *J* = 8.5, 14 Hz, H-3). ^{13}C-NMR (CD_3OD) δ 75.4 (C-2), 43.8 (C-3), 31.6 (C-4), 158.2 (C-5), 95.9 (C-6), 158.2 (C-7), 95.9 (C-8), 157.7 (C-9), 105.7 (C-10), 132.6 (C-1′), 117.7 (C-2′), 145.9 (C-3′), 144.4 (C-4′), 116.1 (C-5′), 121.8 (C-6′). ESI-MS *m/z* 273 $[M - H]^-$.

Procyanidin B-1 (3): off-white amorphous powder. ^{1}H-NMR (CD_3OD) δ 6.69–6.98 (aromatic-H), 6.07 (brs, H-8), 5.94 (2H, brs, H-6, 6′), 5.10 (brs, H-2), 4.96 (brs, H-2′), 4.60 (brs, H-4), 4.06 (brs, H-3′), 3.94 (brs, H-3), 2.70 (m, H-4′), 2.55 (m, H-4′). ESI-MS *m/z* 577 $[M - H]^-$.

(+)-Catechin (4): off-white amorphous powder. $[\alpha]^{27}_D$ +6.9° (*c* 1.0, MeOH). ^{1}H-NMR (CD_3OD) δ 6.83 (1H, d, *J* = 2 Hz, H-2′), 6.75 (1H, d, *J* = 8 Hz, H-5′), 6.70 (1H, dd, *J* = 2, 8 Hz, H-6′), 5.92, 5.85 (each 1H, d, *J* = 2 Hz, H-6, 8), 4.56 (1H, d, *J* = 7.5 Hz, H-2), 3.97 (1H, m, H-3), 2.83 (1H, dd, *J* = 5.5, 16 Hz, H-4), 2.50 (1H, dd, *J* = 8, 16 Hz, H-4). ESI-MS *m/z* 289 $[M - H]^-$.

Vanillic acid (5): off-white amorphous powder. ^{1}H-NMR (CD_3OD) δ 7.55 (1H, d, *J* = 2 Hz, H-2), 7.52 (1H, dd, *J* = 2, 8 Hz, H-6), 6.80 (1H, d, *J* = 8 Hz, H-5), 3.88 (3H, s, -OCH_3). ESI-MS *m/z* 167 $[M - H]^-$.

Procyanidin B-2 (6): off-white amorphous powder. ^{1}H-NMR (CD_3OD) δ 6.60–7.00 (aromatic-H), 6.00 (brs, H-8), 5.90 (2H, brs, H-6, 6′), 5.05 (brs, H-2), 4.95 (brs, H-2′), 4.65 (brs, H-4), 4.25 (brs, H-3′), 3.90 (brs, H-3), 2.95 (m, H-4′), 2.75 (m, H-4′). ESI-MS *m/z* 577 $[M - H]^-$.

Syringic acid (7): light brown amorphous powder. ^{1}H-NMR (CD_3OD) δ 7.32 (2H, s, H-2, 6), 3.87 (6H, s, -OCH_3). ESI-MS *m/z* 197 $[M - H]^-$.

(−)-Epicatechin (8): off-white amorphous powder. $[\alpha]^{27}_D$ −25.2° (*c* 0.5, MeOH). ^{1}H-NMR (CD_3OD) δ 6.97 (1H, d, *J* = 2 Hz, H-2′), 6.79 (1H, dd, *J* = 2, 8.5 Hz, H-6′), 6.75 (1H, d, *J* = 8.5 Hz, H-5′), 5.94, 5.91 (each 1H, d, *J* = 2 Hz, H-6, 8), 4.80 (1H, brs, H-2), 4.12 (1H, m, H-3), 2.85 (1H, dd, *J* = 4.5, 16.5 Hz, H-4), 2.71 (1H, dd, *J* = 3, 16.5 Hz, H-4). ESI-MS *m/z* 289 $[M - H]^-$.

Ethyl gallate (9): light brown amorphous powder. ^{1}H-NMR (CD_3OD) δ 7.04 (2H, s, H-2, 6), 4.26 (2H, dd, *J* = 7.5, 13.5 Hz, -CH_2-), 1.34 (3H, t, *J* = 7.5 Hz, -CH_3). ESI-MS *m/z* 197 $[M - H]^-$.

Myricetin 3-*O*-β-D-glucoside (10): pale yellow amophous powder. ^{1}H-NMR (CD_3OD) δ 7.29 (2H, s, H-2′, 6′), 6.38, 6.20 (each 1H, d, *J* = 2.5 Hz, H-6, 8), 5.23 (1H, d, *J* = 7.5 Hz, Glc H-1), 3.71 (1H, dd, *J* = 2, 12 Hz, Glc H-6), 3.60 (1H, dd, *J* = 5.5, 12 Hz, H-6), 3.52–3.36 (3H, m, Glc H-2-4), 3.23 (1H, m, Glc H-5). ^{13}C-NMR (CD_3OD) δ 159.0 (C-2), 135.8 (C-3), 179.4 (C-4), 163.0 (C-5), 99.9 (C-6), 166.0 (C-7), 94.7 (C-8), 158.4 (C-9), 105.8 (C-10), 122.0 (C-1′), 110.1 (C-2′, 6′), 146.5 (C-3′, 5′), 138.1 (C-4′), 104.3 (C-1″), 75.7 (C-2″), 78.4 (C-3″), 71.1 (C-4″), 78.2 (C-5″), 62.5 (C-6″). ESI-MS *m/z* 479 $[M - H]^-$.

Quercetin 3-*O*-β-D-glucuronide (11): pale yellow amorphous powder. ^{1}H-NMR (CD_3OD) δ 7.96 (1H, d, *J* = 2.5 Hz, H-2′), 7.44 (1H, dd, *J* = 2.5, 8.5 Hz, H-6′), 6.83 (1H, d, *J* = 8.5 Hz, H-5′), 6.13 (1H, *J* = 2 Hz, H-8), 6.00 (1H, d, *J* = 2 Hz, H-6), 4.98 (1H, d, *J* = 7.5 Hz, Glc H-1), 3.61 (1H, d, *J* = 9.5 Hz, Glc H-5), 3.40-3.55 (3H, m, Glc H-2-4). ^{13}C-NMR (CD_3OD) δ 159.1 (C-2), 135.5 (C-3), 179.3 (C-4), 163.0 (C-5), 99.9 (C-6), 166.0 (C-7), 94.7 (C-8), 158.5 (C-9), 105.7 (C-10), 122.8 (C-1′), 117.3 (C-2′), 146.0 (C-3′), 149.9 (C-4′), 116.0 (C-5′), 123.5 (C-6′), 104.3 (C-1″), 75.4 (C-2″), 77.6 (C-3″), 72.9 (C-4″), 77.1 (C-5″), 172.2 (C-6″). ESI-MS *m/z* 477 $[M - H]^-$.

Quercetin (12): pale yellow amorpous powder. ^{1}H-NMR (CD_3OD) δ 7.73 (1H, d, *J* = 2 Hz, H-2′), 7.62 (1H, dd, *J* = 2, 8.5 Hz, H-6′), 6.87 (1H, d, *J* = 8.5 Hz, H-5′), 6.38, 6.17 (each 1H, d, *J* = 2 Hz, H-6, 8). ESI-MS *m/z* 301 $[M - H]^-$.

Fraction of condensed tannin oligomers: ^{13}C-NMR (CD_3OD) NMR spectrum was shown in Fig. 4.

4. Gel permeation chromatography (GPC) analysis

The condensed tannin fraction of grape skin extract (1 mg) was dissolved in *N*,*N*-dimethylformamide (1 mL), and then subjected to GPC analysis. Analytical condition was as follows: column, TSK-gel Super AW3000 (150 × 6.0 mm i.d.) (Tosoh Co., Tokyo, Japan), mobile phase, *N*,*N*-dimethylformamide (including 0.5% 3 M ammonium formate); column temperature, 35°C; flow-rate, 0.3 mL/min; detection, 280 nm. Polystyrene standard (Shodex standard; SL-105, MW 580, 1310, 2970, 5030, 10700, 19900) were used for calibration between retention volume and molecular weight (log MW).

5. Determination of the proanthocyanidin content

To a solution of the grape skin extract (10 mg) in methanol (1 mL) was added 1% vanillin/methanol solution (2 mL) and 25% sulfuric acid/methanol (2 mL). After sonication for 10 min, methanol (1 mL) was added, and the supernatant after centrifugation (6,200 rpm, 3 min) was used as a sample solution. Proanthocyanidin content was determined by colorimetric determination using the spectrophotometer at 500 nm, and the amount of proanthocyanidin in the sample was estimated from the standard curve of similarly treated (+)-catechin.

Ⅲ Results and Discussion

To characterize the constituents in grape skin extract, it was analyzed by HPLC. The HPLC chromatogram (at 280

nm) of an aqueous methanol solution of grape skin extract is shown in Fig. 1 (a). To further characterize the constituents corresponding to the peaks in the HPLC chromatogram, an aqueous solution of the extract was further partitioned into ethyl acetate-, *n*-butanol-, and water-soluble fractions. These HPLC chromatograms were shown in Fig. 1 (b-l). The ethyl acetate fraction was separated by repeated column chromatography with porous polymer gels to give six constituents [luteoliflavan (2), procyanidin B-1 (3), (+)-catechin (4), procyanidin B-2 (6), (−)-epicatechin (8), and myricetin 3-*O*-glucoside (10)]. Similarly, six compounds were isolated from the *n*-butanol fraction and characterized as tryptamine (1), vanillic acid (5), syringic acid (7), ethyl gallate (9), quercetin 3-*O*-glucuronide (11), and quercetin (12). These compounds (Fig. 2) were identified based on their spectroscopic comparison with those reported in the literature,

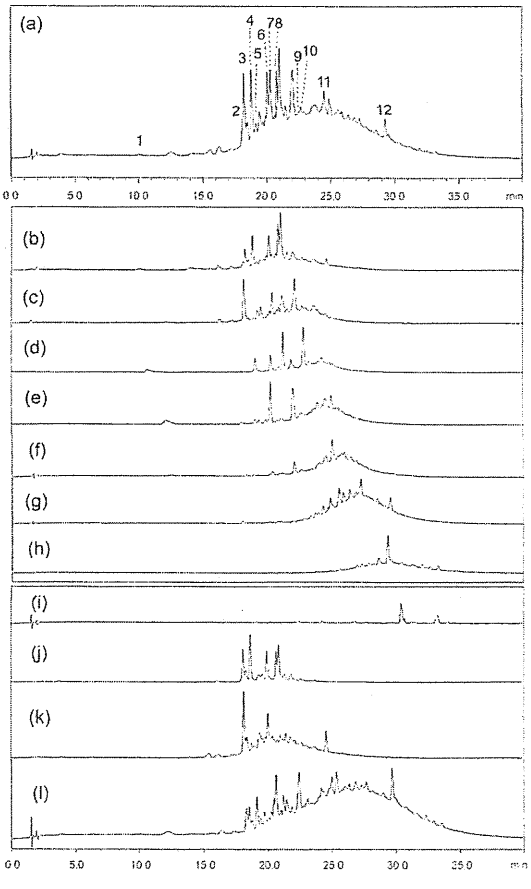

Fig. 1. HPLC chromatograms of grape skin extracts
(a) grape skin extract; (b) Diaion HP-20 [D]-H_2O eluate; (c) D-10% MeOH eluate; (d) D-20% MeOH eluate; (e) D-30% MeOH eluate; (f) D-40% MeOH eluate; (g) D-50% MeOH eluate; (h) D-MeOH eluate; (i) D-H_2O eluate-*n*-hexane fraction; (j) D-H_2O-ethyl acetate fraction; (k) D-H_2O-*n*-BuOH fraction; (l) D-H_2O-H_2O fraction.
The number on the chromatogram corresponds to the compound number. HPLC conditions are described in condition 1 of the "Materials and Methods".

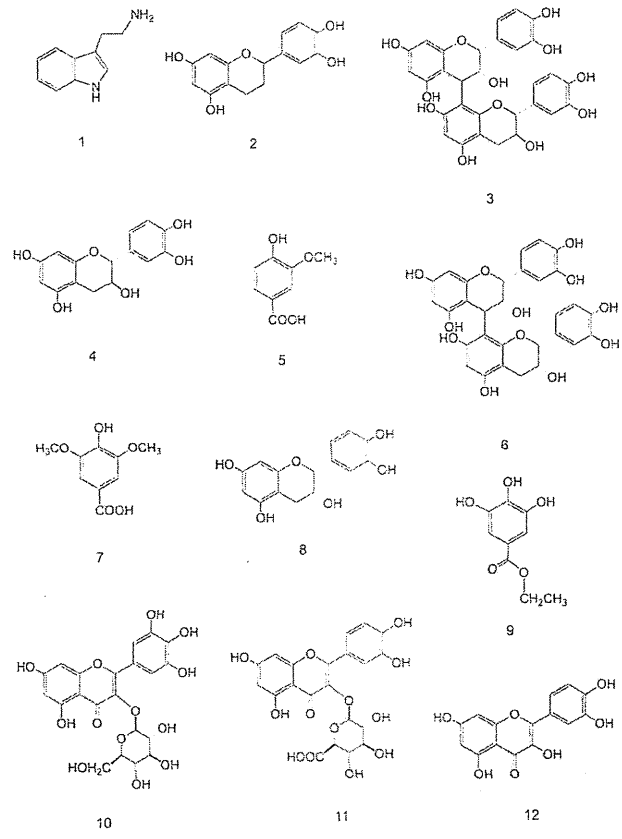

Fig. 2. Structures of compounds 1-12
1, tryptamine; 2, luteoliflavan; 3, procyanidin B-1; 4, (+)-catechin; 5, vanillic acid; 6, procyanidin B-2; 7, syringic acid; 8, (−)-epicatechin; 9, ethyl gallate; 10, myricetin 3-*O*-glucoside; 11, quercetin 3-*O*-glucuronide; 12, quercetin.

or by direct comparison with authentic samples. These compounds are reported as constituents of natural grape skin, and the data obtained from these additive samples was in accordance with that of the natural product.

In addition, as shown in Fig. 3, grape skin anthocyanins, were detected by HPLC, with malvidin 3-*O*-glucoside as the major component, together with the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin. These components were also reported in the natural grape skin[1, 19].

A swollen broad peak of condensed tannins in the HPLC chromatogram of the extract was detected mainly in the chromatogram of the water fraction as shown in Fig. 1 (l). A condensed tannin-rich fraction was separated using an MCI GEL CHP20P column. A fraction of condensed tannin oligomers, which showed only the broad peak, and not the other sharp peaks, was obtained. ^{13}C-NMR analysis indicated the trend of oligomeric B type proanthocyanidins (Fig. 4). This fraction was also subjected to GPC analysis of the molecular weight distribution. A calibration curve based on retention times correlated with the molecular weight of a polystyrene standard allows the assignment of the number and

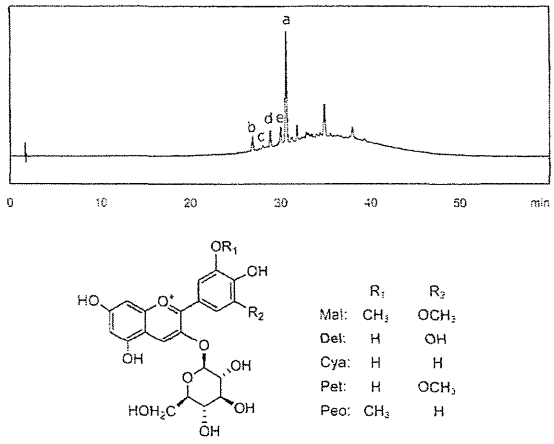

Fig. 3. HPLC chromatogram and structures of anthocyanins in grape skin extracts
a, malvidin 3-*O*-glucoside (Mal); b, delphinidin 3-*O*-glucoside (Del); c, cyanidin 3-*O*-glucoside (Cya); d, petunidin 3-*O*-glucoside (Pet); e, peonidin 3-*O*-glucoside (Peo).

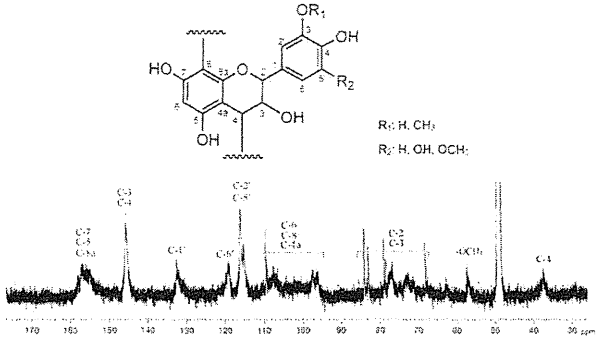

Fig. 4. ^{13}C-NMR spectrum of the condensed tannin oligomer fraction isolated from grape skin extract

weight averaged molecular weights of the concerned fraction as 5999.6 and 21287.7, respectively. Proanthocyanidins have been reported to be the main constituents of grape skin[20]. However, the proanthocyanidin contens of grape skin extract has not been fully investigated. Thus, there is little considered in the analysis of grape skin extract products. In this study, colorimetric analysis of the proanthocyanidins in the extract is performed to provide a simple method for quantifying these components of the extract. The proanthocyanidin contents of two commercial grape skin extract products were 62.1% and 60.7%, respectively.

In summary, we investigated in detail the constituents of grape skin extract, and isolated 12 compounds, as well as a condensed tannin oligomer fraction. The data for this additive product was in close accordance with that of the natural product. It was found by ^{13}C-NMR study that a fraction of condensed tannin oligomer consisted of mainly a B type proanthocyanidins, the molecular weight of which was estimated as a number average of 5999.6 and a weight average of 21287.7 by GPC analysis. The proanthocyanidin content in two commercial grape skin extract products was determined to be around 60% by colorimetric analysis. In this study, it was indicated that compounds isolated and identified were mostly flavan 3-ols and their oligomers. Therefore, it is suggested that determination methods for proanthocyanidins are suitable for the analysis of grape skin extract products.

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