

Analysis of Proanthocyanidins in Grape Seed Extracts, Health Foods and Grape Seed Oils

Yumiko Nakamura,* Sumiko Tsuji, and Yasuhide Tonogai

Division of Food Chemistry, National Institute of Health Sciences, Osaka Branch 1-1-43, Hoenzaka, Chuo-ku, Osaka 540-0006, Japan

(Received August 13, 2002; Accepted September 20, 2002)

Grape seed extract (GSE) is one of Japan's natural food additives, and is recommended for use as an antioxidant. The component of interest is polyphenol, mainly proanthocyanidins, which are condensed tannins. A vanillin-hydrochloric acid (HCl) assay is specific for flavan-3-ol. The sensitivity of GSE components to the vanillin-HCl assay differed according to the chemical structure. We applied this assay to the determination of proanthocyanidin content in GSEs, health foods containing GSEs and grape seed oils. The concentration of proanthocyanidins in GSEs and health foods was overestimated in the vanillin-HCl assay when (+)-catechin was used as a standard. However, the concentration of proanthocyanidins in health foods with high protein or a high colored substance content was underestimated. No proanthocyanidins were detected in grape seed oil. Furthermore, we examined the content of low molecular weight compounds in GSE by reversed-phase HPLC. The percentage of low molecular weight compounds, including gallic acid, (+)-catechin, (-)-epicatechin, procyanidins B1, B2 and C was estimated to be 5.5–12.2% (w/w).

Key words — grape seed extract, proanthocyanidins, vanillin-hydrochloric acid assay, reversed-phase HPLC, health food, grape seed oil

INTRODUCTION

Grape seed extract (GSE) is a natural food additive approved in Japan. Its standard usage is as an antioxidant in confectionery or processed fishery

foods at a concentration of 0.01–1.0%. In Japan, GSE is used as a health food material rather than as a food additive (by private communication with Mr. Asano at Japan Food Additives Association). The consumption of GSE in Japan is approximately 100000 kg/year.¹⁾ The biologically active constituents of GSE are proanthocyanidins (Fig. 1), which represent a variety of polymers of flavan-3-ol, such as catechin and epicatechin^{2–5)} and have a strong antioxidative effect in aqueous systems.^{6–8)}

Colorimetric procedures such as the vanillin-hydrochloric acid (HCl) assay,^{9–11)} butanol-HCl assay,¹²⁾ and the Folin-Ciocalteu assay¹³⁾ have been generally used for the determination of proanthocyanidins in foodstuffs. The vanillin-HCl assay is specific for a narrow range of flavanols and dihydrochalcones that have a single bond at the 2,3 position and free *m*-oriented hydroxy groups on the B ring.⁹⁾ The principle of the vanillin-HCl assay is as follows: vanillin is protonated in an acid solution, giving a weak electrophilic carbocation that reacts with the flavonoid ring at the 6 or 8 position. This intermediate compound is dehydrated to give a red colored compound.¹¹⁾

In this study, we applied the vanillin-HCl assay proposed by Sun *et al.*⁹⁾ to the determination of proanthocyanidin content in GSEs, health foods containing GSE and grape seed oils consumed in Japan. We also determined the content of some low molecular weight compounds by reversed-phase HPLC.

MATERIALS AND METHODS

Materials — Gallic acid (GA), vanillin and flavone were purchased from Wako Pure Industries, Ltd. (Osaka, Japan). (+)-Catechin (CT) (purity > 98%), (-)-epicatechin (EC), procyanidin B1 (ProB1), procyanidin B2 (ProB2) and procyanidin C1 (ProC1) were purchased from Funakoshi (Tokyo, Japan). Standards of other flavonoids and phenolic compounds were purchased from Funakoshi or Extrasynthèse (Geney, France). 1,2,3,6-Tetra-O-galloyl β -D-glucose (TGG) and 1,2,3,4,6-penta-O-galloyl β -D-glucose (PGG) were kindly given by Prof. T. Yoshida (Faculty of Pharmaceutical Sciences, Okayama University). Ion-exchanged and redistilled water was used throughout the experiments. HPLC grade acetonitrile, ethyl acetate, *n*-hexane and methanol were used for analyses. Other reagents were analytical grades.

*To whom correspondence should be addressed: Division of Food Chemistry, National Institute of Health Sciences, Osaka Branch 1-1-43, Hoenzaka, Chuo-ku, Osaka 540-0006, Japan. Tel.: +81-6-6941-1533; Fax: +81-6-6942-0716; E-mail: ynakamura@nihs.go.jp

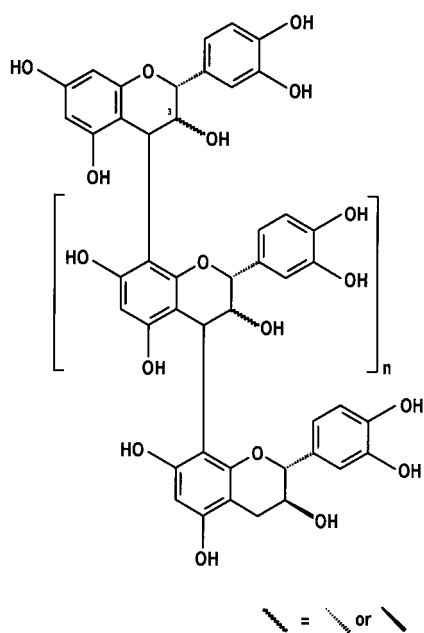


Fig. 1. Chemical Structure of Proanthocyanidins

The HPLC column Develosil 300 ODS-HG-5 (4.6 mm i.d. \times 250 mm) was purchased from Nomura Chemical Co., Ltd. (Seto, Japan).

All the reagents for the vanillin-HCl assay were prepared immediately before use. A standard stock solution of CT for the vanillin-HCl assay was prepared by dissolving 10 mg of CT in 10 ml of methanol. The final solution of CT (20–300 $\mu\text{g}/\text{ml}$) for the vanillin-HCl assay was prepared by diluting the stock solution with adequate amounts of methanol. Vanillin was dissolved in methanol to give a 1% (w/v) vanillin solution. Concentrated HCl was dissolved in methanol to give a 9 mol/l HCl solution.

Apparatus — A Hitachi U-3210 spectrophotometer was used for the vanillin-HCl assay. A Shimadzu HPLC apparatus, Class-VP Series (Kyoto, Japan), equipped with system controller SCL-10AVP, pump liquid chromatography (LC)-10ADVP, degasser DGU-14A, auto injector SIL-10ADVP, column oven CTO-10AC and diode array detector SPD-M10AVP, was used to analyze the low molecular weight compounds in GSEs.

Samples — GSEs #1–4 were obtained from the Japan Food Additives Association via Dr. T. Maitani (National Institute of Health Sciences). GSE #5 was purchased from a domestic manufacturer. GSEs #1 and #2, GSEs #4 and #5 are the same products but differ in batch number. Health foods containing GSE and the grape seed oils were purchased from retail markets in Osaka Prefecture.

Ten mg of each GSE was weighed and dissolved in methanol, and the volume was adjusted to 50 ml (sample solution). Tablets of the health food were ground to a powder using a mill. The powder of each health food was weighed and dissolved in methanol to a final concentration of 150–250 $\mu\text{g}/\text{ml}$ of proanthocyanidins (sample solution). The centrifugation process (3000 rpm \times 15 min) was performed if necessary and the supernatant was used in the vanillin-HCl assay. The lipid in 1 g of grape seed oil was removed by a *n*-hexane/acetonitrile partition, which was repeated three times. Acetonitrile fractions were jointed, evaporated to dryness and dissolved in 2 ml of methanol (sample solution).

Vanillin-HCl Assay — Proanthocyanidins in the sample solutions of 5 GSEs, 4 health foods and 2 grape seed oils were determined by the vanillin-HCl assay described by Sun *et al.*⁹⁾ To 1 ml of CT solution (0–300 $\mu\text{g}/\text{ml}$ in methanol) or test solution (150–250 $\mu\text{g}/\text{ml}$ polyphenols in methanol) in a test tube, 2.5 ml of methanol (control) or 1% vanillin solution in methanol (sample) and 2.5 ml of 9 mol/l HCl in methanol was added. The reaction mixture was incubated for 20 min at 30°C and the absorbance at 500 nm was measured. The following A_0 , A_b , A_c , A_s was measured for each standard and sample.

A_0 = Absorbance at 500 nm of the control of 0 mg CT [1 ml methanol + 2.5 ml methanol + 2.5 ml 9 mol/l HCl].

A_b = sample of 0 mg CT [1 ml methanol + 2.5 ml 1% vanillin solution + 2.5 ml 9 mol/l HCl].

A_c = control [1 ml CT (20–300 $\mu\text{g}/\text{ml}$) or test solution + 2.5 ml methanol + 2.5 ml 9 mol/l HCl].

A_s = sample [1 ml CT (20–300 $\mu\text{g}/\text{ml}$) or test solution + 2.5 ml 1% vanillin + 2.5 ml 9 mol/l HCl].

A was calculated as follows for each standard and sample solution:

$$A = (A_s - A_b) - (A_c - A_0)$$

A calibration curve was prepared using A for the CT solution using the above calculation. Total flavan-3-ol in each test solution was calculated from the calibration curve.

The Vanillin-HCl assay was performed 3–5 times for each sample.

Reversed-Phase HPLC — HPLC was performed as follows: apparatus, Shimadzu Class VP series; column, Develosil 300 ODS-HG-5; column oven temperature, 35°C; mobile phase, (A) water/phosphoric acid 1000 : 1 (v/v), (B) acetonitrile/phosphoric acid 1000 : 1 (v/v); gradient program B, 0% (0 min) \rightarrow 15% (20 min) \rightarrow 30% (25 min) \rightarrow 100% (45–61 min) \rightarrow 0 (62–77 min); a fluorescence de-

Table 1. Reactivity of Various Polyphenol to Vanillin-HCl Assay^{a)}

Compounds Trivial name	Category	Molecular weight	Vanillin-HCl method			
			Reactivity ^{b)}	λ_{\max} [nm]	Sensitivity (Catechin as 1.000)	
				per mg	per μmol	
Flavone	Flavone	222.2	×	—	—	—
Diosmetin	Flavone	300.3	×	—	—	—
Quercetin	Flavonol	302.2	×	—	—	—
Rutin	Flavonol <i>O</i> -glycoside	610.5	×	—	—	—
Genistein	Isoflavone	270.2	×	—	—	—
Daidzein	Isoflavone	254.2	×	—	—	—
Formononetin	Isoflavone	268.3	×	—	—	—
Biochanin A	Isoflavone	284.3	×	—	—	—
Genistin	Isoflavone <i>O</i> -glycoside	432.4	×	—	—	—
Daidzin	Isoflavone <i>O</i> -glycoside	416.4	×	—	—	—
(+)-Catechin	Flavan-3-ol	290.3	○	498.6	1.000	1.000
(-)-Epicatechin	Flavan-3-ol	290.3	○	499.2	1.057	1.057
Procyanidin B1	Procyanidin dimer	578.5	○	499.4	1.683	3.354
Procyanidin B2	Procyanidin dimer	578.5	○	499.4	1.747	3.482
Procyanidin C1	Procyanidin trimer	866.8	○	501.0	1.533	4.577
(-)-Gallocatechin	Flavan-3-ol	306.3	○	504.4	0.449	0.474
(-)-Epigallocatechin	Flavan-3-ol	306.3	○	500.0	0.892	0.941
(-)-Catechin gallate	Flavan-3-ol	442.4	○	504.0	0.368	0.561
(-)-Epicatechin gallate	Flavan-3-ol	442.4	○	503.2	0.520	0.792
(-)-Gallocatechin gallate	Flavan-3-ol	458.4	○	504.0	0.344	0.544
(-)-Epigallocatechin gallate	Flavan-3-ol	458.4	○	502.0	0.431	0.680
Gallic acid	Phenolic acid	171.1	×	—	—	—
Ellagic acid	(Phenolic acid)	302.2	×	—	—	—
1,2,3,6-tetra- <i>O</i> -galloyl- β -D-glucose	Gallotannin	788.6	×	—	—	—
1,2,3,4,6-penta- <i>O</i> -galloyl- β -D-glucose	Gallotannin	940.7	×	—	—	—
Tannic acid	Gallotannin		×	—	—	—
Grape seed extract #1	Grape seed polyphenol		○	503.8	0.482	—
Grape seed extract #2	Grape seed polyphenol		○	504.4	0.473	—
Grape seed extract #3	Grape seed polyphenol		○	499.8	0.970	—
Grape seed extract #4	Grape seed polyphenol		○	502.8	1.118	—
Grape seed extract #5	Grape seed polyphenol		○	500.2	1.023	—
Polyphenon-100	Green tea polyphenol		○	500.8	0.778	—

a) Details for vanillin-HCl assay are described in the text. b) ○: reacted to the vanillin-HCl assay; ×: not reacted to the vanillin-HCl assay.

tector, ex 283 nm, em 317 nm for CT, EC, ProB1, ProB2 and ProC2; diode array detector (DAD), monitoring wavelengths, 210 nm; injection volume, 10 μl .

Phosphoric acid has an absorbance at 210 nm, therefore the HPLC chromatograms were corrected by subtracting the base line.

RESULTS AND DISCUSSION

Vanillin-HCl Assay

The detection limit of the vanillin-HCl assay described here was 5 μg CT. The linear range of the calibration curve for the vanillin-HCl assay was 10–400 $\mu\text{g}/\text{ml}$ ($r^2 = 0.998$).

The reactivity of various polyphenols in the vanillin-HCl assay is shown in Table 1. Reaction in the vanillin-HCl assay (shown by the presence of a red compound) was detected in flavan-3-ol, procyanidin

Table 2. Relative Retention Times and Detection Limits of Polyphenols

Polyphenol ^{a)}	Molecular weight	Relative retention time ^{b)} (flavone = 1.00) (determined by DAD)	Detection limit (S/N = 3) [pmol/ml]		Linear range of calibration curve [pmol/ml] (r ²)	
			Fluorescence (Ex 283 nm, Em 317 nm)	Ultraviolet (210 nm except 260 nm for EA)	Fluorescence (Ex 283 nm, Em 317 nm)	Ultraviolet (210 nm except 260 nm for EA)
CT	290.3	0.507	33.9	170	67.8–13560 (1.000)	167–13560 (0.999)
EC	290.3	0.607	34.6	173	69.2–13836 (1.000)	173–13836 (0.999)
ProB1	578.5	0.467	83.4	167	83.4–7468 (0.998)	167–7468 (0.997)
ProB2	578.5	0.568	47.6	238	47.6–9510 (1.000)	119–9510 (0.998)
ProC1	866.8	0.649	37.3	187	37.3–7468 (1.000)	187–7468 (0.998)
GA	170.1	0.239		532		532–21260 (0.999)
Flavone (IS)	222.2	1.000				

Data are the averages for 3–5 trials. *a)* Abbreviations are: CT, (+)-catechin; EC, (–)-epicatechin; ProB1, procyanidin B1; ProB2, procyanidin B2; ProC1, procyanidin C1; GA, gallic acid. *b)* Conditions for HPLC are as follows: Apparatus, Shimadzu Class VP series (pump LC-10ADVP + degasser DGU-14A + system controller SCL-10ADVP + autoinjector 10ADVP + column oven CTO-10ACVP + diode array detector (DAD) SPD-M10AVP + fluorescence detector RF-10AXL); column, Develosil 300 ODS-HG-5 (ϕ 4.6 mm \times 250 mm); column oven temperature, 35°C; mobile phase, (A) water/phosphoric acid 1000 : 1 (v/v), (B) acetonitrile/phosphoric acid 1000 : 1 (v/v); gradient program B, 0% (0 min) \rightarrow 15% (20 min) \rightarrow 30% (25 min) \rightarrow 100% (45–61 min) \rightarrow 0 (62–77 min); fluorescence detector, ex 283 nm, em 317 nm for CT, EC, ProB1, ProB2 and ProC2; DAD, monitoring wavelength, 210 nm; injection volume, 10 μ l.

oligomers, grape seed polyphenols and green tea polyphenol. Flavone, flavonol, isoflavone, the glycosides of flavanol and isoflavone, phenolic acid and gallotannin did not react in the vanillin-HCl assay (Table 1). Differences in the sensitivity to the vanillin-HCl assay were observed; higher reactivity was observed in procyanidins which are highly polymerized and lower reactivity was observed in catechins which are highly esterified by GA. In addition, EC was more reactive than CT (Table 1). Sarkar *et al.*¹¹⁾ showed that dihydrocalcones also react in the vanillin-HCl assay and that a weak reactivity was observed for flavanones. A higher sensitivity to the vanillin-HCl assay was detected in proanthocyanidins which have a higher molecular weight.⁹⁾ Sun *et al.*⁹⁾ investigated the various parameters of the vanillin-HCl assay and showed that when the water content was high, reactivity in the vanillin-HCl assay was low. Due to the high sensitivity and low differences in the determined value when there is a large difference in the degree of polymerization, they recommend the vanillin-sulfuric acid assay instead of the vanillin-HCl assay. We used the vanillin-HCl method because of its simplicity.

Analysis of GSE

Reversed-phase HPLC,^{2,4,14–16)} normal-phase HPLC,¹⁷⁾ and the LC/MS^{17–20)} have been used in the analysis of GSE. It has been suggested that complete separation of all GSE components by chromatographic methods is very difficult because polyphenol has a large number of hydroxyl groups and there

are many kinds of isomers.

The pore size of an ordinary reversed-phase HPLC column is about 100 Å. Using this size column it is possible to determine low molecular weight compounds (less than 1000), but higher molecular weight compounds are not eluted. GSEs include compounds of high molecular weight (greater than 1000), therefore, despite lower sensitivity, the Develosil 300 ODS-HG-5 column, which has a pore size of 300 Å, was used to determine all compounds in GSEs.

Relative retention time (flavone = 1.00), detection limits and the linear range of the calibration curve by HPLC are shown in Table 2. CT, EC, ProB1, ProB2 and ProC1 could be determined separately and sensitively by fluorescence detection.

The amount of GA, CT, EC, ProB1, ProB2 and ProC1 (Fig. 2) in each GSE ($n = 5$ for each sample) are shown in Table 3. The amount of GA in GSEs #1 and #2 was 2.0–2.1% (w/w) whereas the amount of GA in GSEs #3, #4 and #5 was below 0.1% (w/w). This might be due to differences in the manufacturing process. Tannase was used in the manufacturing process of GSEs #1 and #2, which may increase the GA content. The amount of the 6 compounds in GSEs #1 and #2 was low (5.46–5.68%) compared to that in GSEs #3–5 (9.59–12.2%), which reflects the purity of the GSEs (Table 3).

HPLC chromatograms of the sample solution of GSEs #1 and #5 are shown in Figs. 3 and 4. All the HPLC chromatograms have a broad peak after 28 min of retention time. This broad peak was not

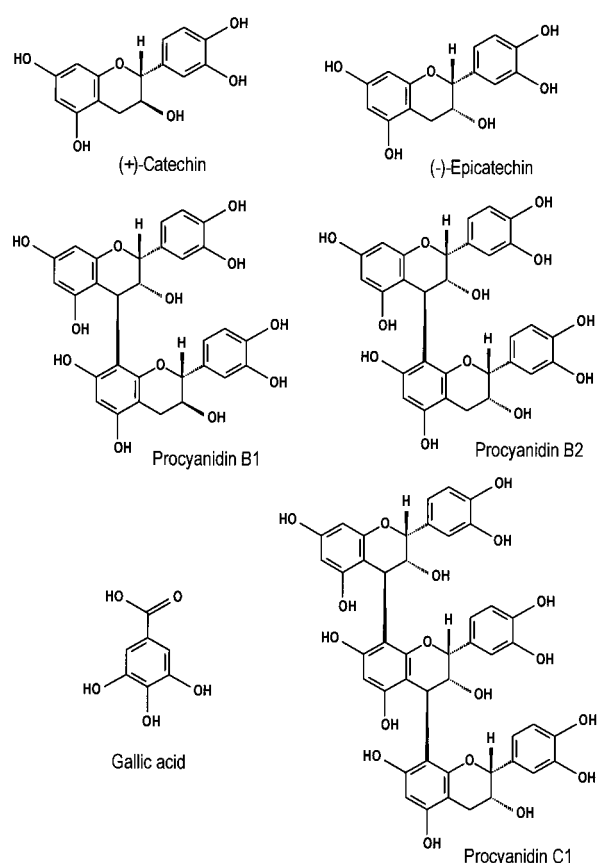


Fig. 2. Chemical Structure of the Low Molecular Weight Compounds in the GSE

separated by reversed-phase HPLC and it is suggested that it is a mixture of higher molecular weight proanthocyanidins. The relative sensitivity of this broad peak to low molecular weight compounds (CT,

EC, ProB1, ProB2 and ProC1) was lower in fluorescent detection than UV detection (Figs. 3 and 4). Molecular weight-dependent chromatograms of GSE sample solution could not be obtained probably because of the existence of many kinds of isomers in GSE (data are not shown).

The amount of total flavan-3-ol in each GSEs determined by the vanillin-HCl assay ($n = 5$) is also shown in Table 3. GSEs #1 and #2 were not completely dissolved in methanol. The trend in composition of each component analyzed in the GSEs was in agreement with the values given by the manufacturers; however, we obtained higher values overall for each component. The broad peak in HPLC chromatogram (Figs. 3 and 4) seemed to contribute mainly in the amount of total flavan-3-ol.

Analysis of Health Foods and Grape Seed Oils

Table 4 shows the amount of procyanidins measured as total flavan-3-ol in four health foods and two grape seed oils, as determined by the vanillin-HCl assay. Methanol-undissolved components were detected in the sample solutions of health foods A and B and the amount of flavan-3-ol exceeded that given by the manufacturer. In the sample solutions of health foods C and D, the amount of procyanidins was less than that given by the manufacturer. Health food C has a high protein content and health food D contains a high concentration of colored substances. In the manufacturing process, proanthocyanidins might bind to proteins to form an insoluble complex; it is suggested that it is then difficult to extract proanthocyanidins with methanol from this undis-

Table 3. Composition of GSE

	GSE #1	GSE #2	GSE #3	GSE #4	GSE #5
Polyphenols of the low molecular weight determined by reversed-phase HPLC ^(a) [% (w/w)]					
Gallic acid	2.06 ± 0.05	2.00 ± 0.08	0.08 ± 0.02	0.02 ± 0.03	0.14 ± 0.01
(+)-Catechin	1.03 ± 0.34	1.06 ± 0.35	4.13 ± 0.32	4.93 ± 0.26	3.24 ± 0.15
(-)-Epicatechin	0.61 ± 0.20	0.63 ± 0.20	2.43 ± 0.19	2.83 ± 0.08	2.06 ± 0.12
Procyanidin B1	0.70 ± 0.08	0.69 ± 0.03	1.42 ± 0.15	1.73 ± 0.08	1.54 ± 0.10
Procyanidin B2	0.66 ± 0.08	0.74 ± 0.13	0.93 ± 0.11	1.54 ± 0.12	1.35 ± 0.05
Procyanidin C1	0.40 ± 0.11	0.57 ± 0.19	0.96 ± 0.17	1.16 ± 0.36	1.26 ± 0.52
Total	5.46 ± 0.66	5.68 ± 0.40	9.95 ± 0.64	12.20 ± 0.71	9.59 ± 0.76
Total flavan-3-ol ^(b) [%]	48.2 ± 0.2	47.3 ± 1.2	111.8 ± 1.1	97.0 ± 0.9	102.3 ± 0.6
Values determined by an autonomous standard method					
Total flavanol	44.5%	44.5%	96.5%	92.2%	99.0%
Proanthocyanidin	42.8%	42.8%	87.7%	Not indicated	Not indicated

Data are Means ± S.D. for 5 trials. ^{a)} HPLC conditions are described in the legend to Table 2. ^{b)} Total flavan-3-ol was analyzed by the vanillin-HCl assay, details of which are described in the legend to Table 1.

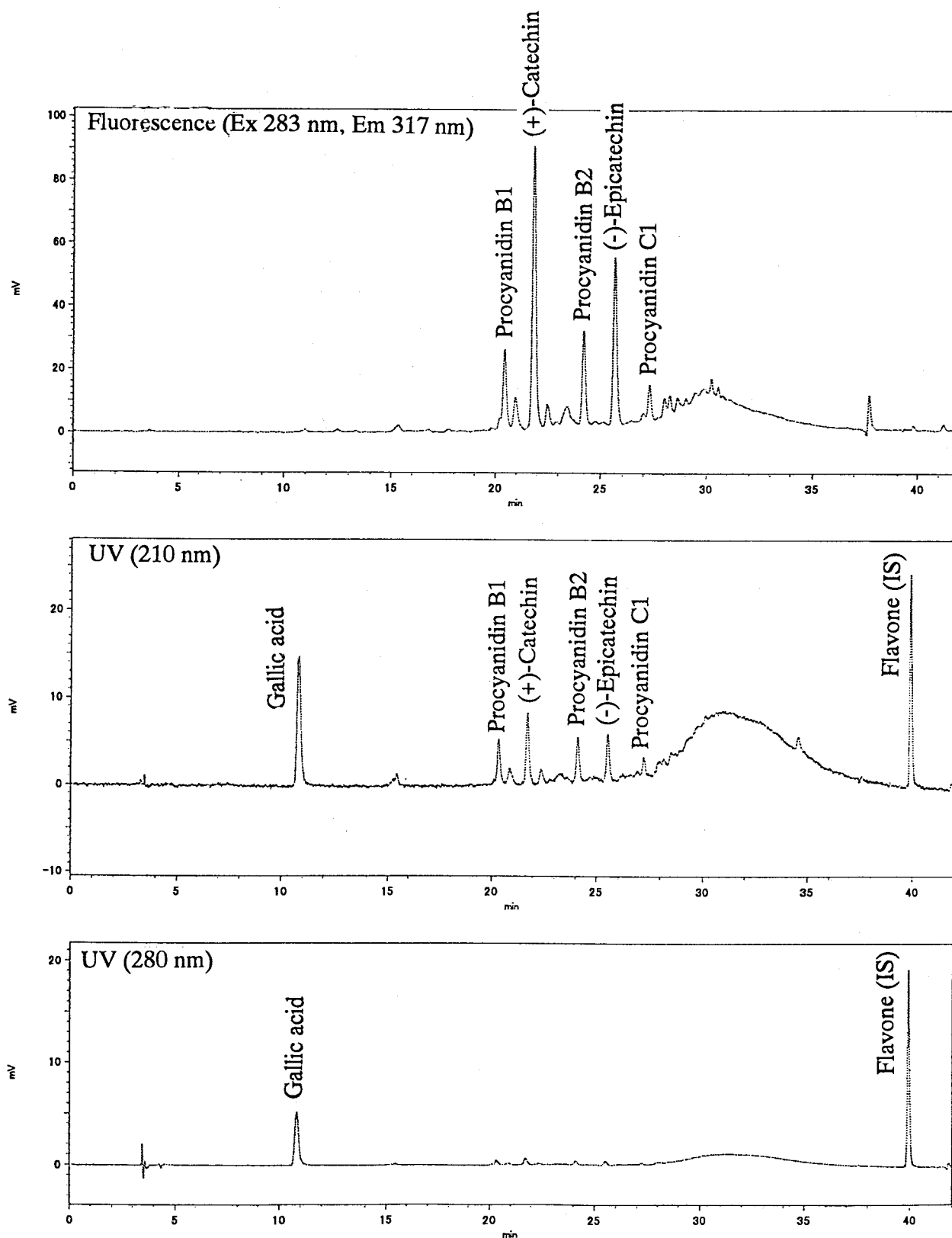


Fig. 3. HPLC Chromatogram of GSE #1 Sample Solution

Sample solution of GSE #1 (200 $\mu\text{g/ml}$) was analyzed by HPLC. HPLC conditions were as follows: column, Develosil 300; column oven temperature, 35°C; mobile phases, (A) water/phosphoric acid 1000 : 1 (v/v), (B) acetonitrile/phosphoric acid 1000 : 1 (v/v); a gradient program, (B) 0% (0 min) \rightarrow 15% (20 min) \rightarrow 30% (25 min) \rightarrow 100% (45–61 min) \rightarrow 0 (62–77 min); detector, DAD; monitor wavelengths, 210 nm and 280 nm; injection volume, 10 μl . Because phosphoric acid has an absorbance at 210 nm, the HPLC chromatograms were corrected by subtracting the base line.

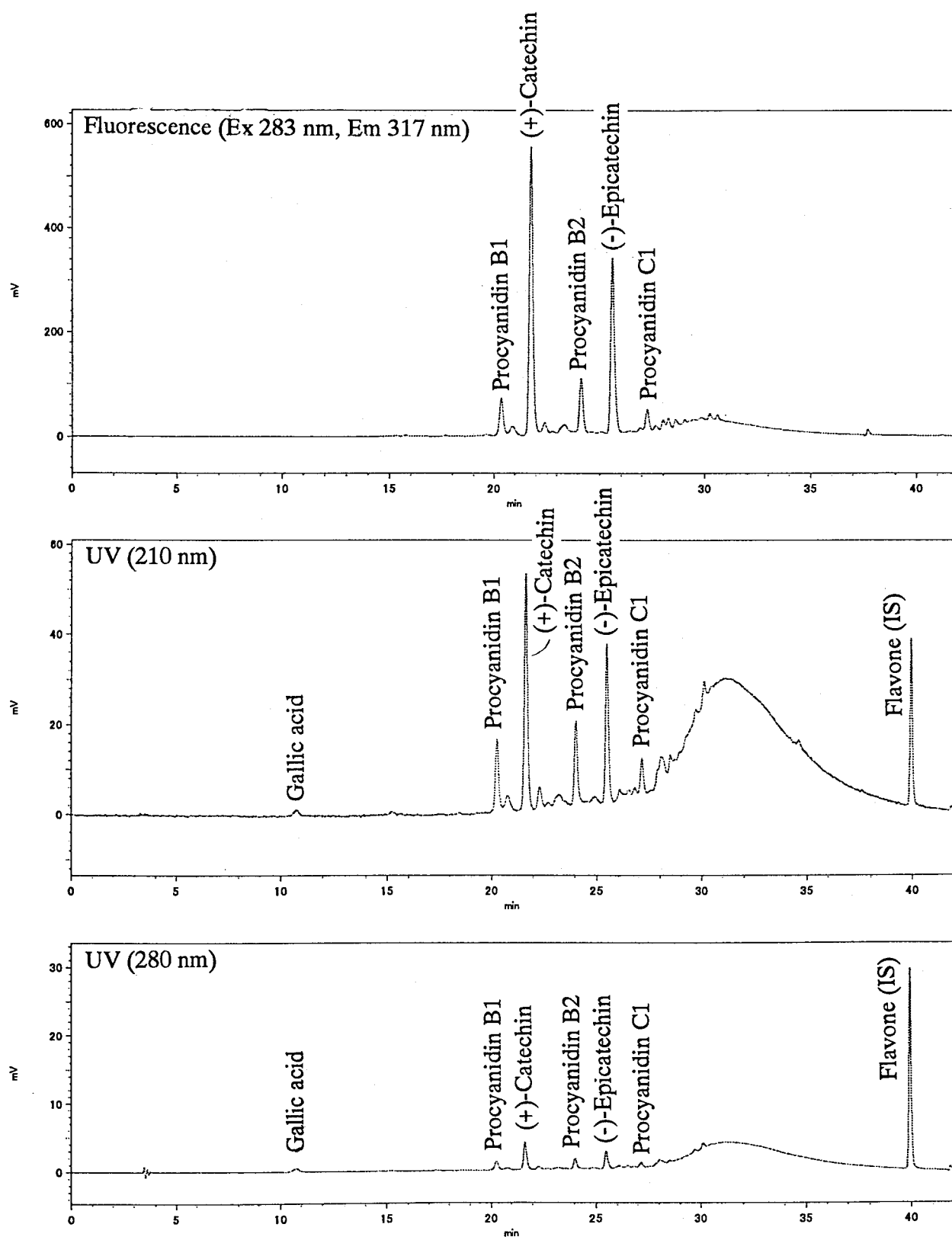


Fig. 4. HPLC Chromatogram of GSE #5 Sample Solution

Sample solution of GSE #5 (200 $\mu\text{g}/\text{ml}$) was analyzed by HPLC. See the footnote of Fig. 4 for further information.

Table 4. Content of Proanthocyanidins in Health Foods

Sample name	Concentrations of proanthocyanidins [mg/g]	Indication by manufacturer	Recommended intake indicated by manufacturer	Usage	Comments on the label
Health food A	239.8 ± 1.5	200 (100 mg/500 mg)	100 mg/day	Tablet 500 mg per 2 tablets	Per 2 tablets (500 mg): Proanthocyanidin 100 mg Na 0.0336% Carbohydrates 90.6% Lipids 4.6% Protein 1.4% 2.05 kcal
Health food B	249.6 ± 3.4	200 (100 mg/500 mg)	100 mg/day	Tablet 500 mg per 2 tablets	Per 2 tablets (500 mg): Proanthocyanidin 100 mg Na 0.0336% Carbohydrates 90.6% Lipids 4.6% Protein 1.4% 2.05 kcal
Health food C	0.683 ± 0.065	10 (1 g/100 g)	no indication	Powder (sticky) Approximately 10 g dissolved in 200 ml milk, apple juice or water.	Per 100 g: Proanthocyanidin 1 g Protein 81 g Lipids 0.3 g Carbohydrates 9.8 g Others (vitamins, minerals) 366 kcal
Health food D	25.46 ± 1.86	33.3 (50 mg/1.5 g)	50 mg/day	Tablet 1.5 g per 6 tablets	Per 6 tablets (1.5 g): grape seed extract 50 mg Melilotus extract 300 mg Citrus extract 300 mg Aloe dry powder 200 mg Fucus extract 120 mg Calcified seaweed 120 mg Marronnier bark extract 100 mg 5.30 kcal
Grape seed oil A	not detected	no indication	no indication	Oil (green colored)	Lipids 100% 900 kcal per 100 g
Grape seed oil B	not detected	no indication	no indication	Oil (yellow-green colored)	Lipids 100% 900 kcal per 100 g

Data are means ± S.D. ($n = 3$). Procyanidins were analyzed by vanillin-HCl assay as total flavan-3-ol. Lipids in the grape seed oil were excluded by the n-hexane/acetonitrile partition method.

solved complex. Flavan-3-ol was not detected in the sample solutions of the grape seed oils E and F.

A recovery test using adequate amount of CT was performed in triplicate using GSE #3, health foods B, C, D and grape seed oil B. The results are shown in Table 5. The recovery of CT from GSE #3 and health food B was 112.5–119.8% and those from health foods C and D, and grape seed oil B was 84.4–89.4%. In case of GSE #3 and health food B, the

content of high molecular proanthocyanidins which has higher reactivity to vanillin-HCl assay than CT was probably higher than the fortified amount of CT, so the recovery of CT might be overestimated. This suggests that the amount of proanthocyanidins (estimated as total flavan-3-ol) in GSE or health foods may be overestimated, and those that contain high concentrations of proteins or colored substances tend to be underestimated using the vanillin-HCl assay.

Table 5. Recovery Test by Vanillin-HCl Assay

Sample name	Concentration of proanthocyanidin ^{a)} [mg/g]	Fortified amount [mg/g]	Recovery ^{b)} [%]
Grape seed extract #3	1118 ± 11	500	112.5 ± 5.0
Health food B	249.6 ± 3.4	100	119.8 ± 10.9
Health food C	0.683 ± 0.065	5	84.4 ± 5.9
Health food D	25.46 ± 1.86	16.7	89.4 ± 9.0
Grape seed oil B	not detected	0.5	84.5 ± 10.6

a) Concentration of proanthocyanidins was measured as flavan-3-ol by the vanillin-HCl assay. Values are means ± S.D. for 3–5 trials. b) Values are means ± S.D. for 3 trials.

Acknowledgements This study was partly supported by a grant from the Ministry of Health and Welfare of Japan in 2001. We are indebted to Prof. T. Yoshida (Faculty of Pharmaceutical Sciences, Okayama University) for the gift of TGG and PGG. We also thank Mr. M. Yanagisawa for his suggestions and support in the GPC analysis.

REFERENCES

- 1) Market view. (2001) Antioxidant foodstuffs. *Nikkei Bio-business*, **2001-11**, 129–130.
- 2) Bartolomé, B., Hernández, T., Bengoechea, M. L., Quesada, C., Gómez-Cordovés, C. and Estrella, I. (1996) Determination of some structural features of procyanidins and related compounds by photodiode-array detection. *J. Chromatogr. A.*, **723**, 19–26.
- 3) De Freitas, V. A. P., Glories, Y. and Laguerre, M. (1998) Incidence of molecular structure in oxidation of grape seed procyanidins. *J. Agric. Food Chem.*, **46**, 376–382.
- 4) Lazarus, S. A., Adamson, G. E., Hammerstone, J. F. and Schmitz, H. H. (1999) High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.*, **47**, 3693–3701.
- 5) Peng, Z., Hayasaka, Y., Iland, P. G., Sefton, M., Ho, P. and Waters, E. J. (2001) Quantitative analysis of polymeric procyanidins (tannins) from grape (*Vitis vinifera*) seeds by reverse phase high-performance liquid chromatography. *J. Agric. Food Chem.*, **49**, 26–31.
- 6) Plumb, G. W., de Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V. and Williamson, G. (1998) Antioxidant properties of catechins and proanthocyanidins: effect of polymerization, galloylation and glycosylation. *Free Rad. Res.*, **29**, 351–358.
- 7) Nuttall, S. L., Kendall, M. J., Bombardelli, E. and Morazzoni, P. (1998) An evaluation of the antioxidant activity of a standardized grape seed extract, Leucoselect®. *J. Clin. Pharm. Ther.*, **23**, 385–389.
- 8) De Gaulejac, N. S.-C., Provost, C. and Vivas, N. (1999) Comparative study of polyphenol scavenging activities assessed by different methods. *J. Agric. Food Chem.*, **47**, 425–431.
- 9) Sun, B., Ricardo-da-Silva, J. M. and Spranger, I. (1998) Critical factors of vanillin assay for catechins and proanthocyanidins. *J. Agric. Food Chem.*, **46**, 4267–4274.
- 10) Goldstein, J. L. and Swain, T. (1963) Changes in tannins in ripening fruits. *Phytochemistry*, **2**, 371–383.
- 11) Sarkar, S. K. and Howarth, R. E. (1976) Specificity of the vanillin test for flavanols. *J. Agric. Food Chem.*, **24**, 317–320.
- 12) Porter, L. J., Hrstich, L. N. and Chan, B. G. (1986) The conversion of proanthocyanidins and prodelfenidins to cyanidins and delphenidins. *Phytochemistry*, **25**, 223–230.
- 13) Singleton, V. L. and Rossi, J. A., Jr. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *Am. J. Enol. Vitic.*, **16**, 144–158.
- 14) Escribano-Bailon, T., Gutierrez-Fernandez, Y., Rivas-Gonzalo, J. C. and Santos-Buelga, C. (1992) Analysis of flavan-3-ol from *Vitis vinifera* variety tempranillo grape seeds. *Bull. Liaison-Grape Polyphenols*, **16** (Pt. 2), 129–132.
- 15) Oszmianski, J., Romeyer, F. M., Sapis, J. C. and Macheix, J. J. (1986) Grape seed phenolics: extraction as affected by some conditions occurring during wine processing. *Am. J. Enol. Vitic.*, **37**, 7–12.
- 16) Revilla, E., Bourzeix, M. and Alonso, E. (1991) Analysis of catechins and proanthocyanidins in grape seeds by HPLC with photodiode array detection. *Chromatographia*, **31**, 465–468.
- 17) Lazarus, S. A., Adamson, G. E., Hammerstone, J. F. and Schmitz, H. H. (1999) High-performance liquid chromatography/mass spectrometry analysis of

- proanthocyanidins in foods and beverages. *J. Agric. Food Chem.*, **47**, 3693–3701.
- 18) Yang, Y. and Chien, M. (2000) Characterization of grape procyanidins using high-performance liquid chromatography/ mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem.*, **48**, 3990–3996.
- 19) Vivas, N., Bourgeois, G., Virty, C., Glories, Y. and de Freitas, V. (1996) Determination of the composition of commercial tannin extracts by liquid secondary ion mass spectrometry (LSIMS). *J. Sci. Food Agric.*, **72**, 309–317.
- 20) Krueger, C. G., Dopke, N. C., Treichel, P. M., Folts, J. and Reed, J. D. (2000) Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.*, **48**, 1663–1667.