Novel Tartaric Acid Isoflavone Derivatives That Play Key Roles in Differentiating Japanese Soy Sauces

Emiko Kinoshita,* Yoshinori Ozawa, and Tetsuo Aishima

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda, Chiba 278, Japan

Nonvolatile components in various brands of Japanese fermented soy sauce were analyzed by gradient reversed phase HPLC. The resulting HPLC profiles were correctly assigned into their original brands by chemometric pattern recognition techniques, such as cluster analysis, linear discriminant analysis (LDA), linear discriminant analysis using genetic algorithm (GALDA), and SIMCA. Three key components found to significantly contribute to the differentiation were isolated by preparative HPLC and purified. Their FAB-MS, ^{1}H- and ^{13}C-NMR, and IR spectra suggested that their molecular weights were 386, 402, and 418, respectively, and that each contained isoflavone moieties. By applying HMBC spectral analysis, these isoflavone derivatives were identified as conjugated ethers of 2,3-dihydroxysuccinic acid (tartaric acid) with daidzein, genistein, and 8-hydroxygenistein. These new isoflavone derivatives are produced during the mash fermentation in the soy sauce manufacturing process.

Keywords: *Daidzein; genistein; new isoflavones; pattern recognition; soy sauce*

INTRODUCTION

Fermented soy sauce is now widely used not only in Asian countries but also in North America and Europe because of its unique appetizing flavor. Authentic soy sauce production takes a long time and requires several steps (Fukushima, 1985). First, soybeans and wheat, starting materials, are steamed and roasted, respectively, so that higher molecular weight proteins and starch can be digested by *Aspergillus oryzae* or *Aspergillus sojae* during the following dry mash or *koji* step and thereafter. During the *koji* period for 2−3 days, proteins, starch, and other higher molecular weight compounds contained in soybeans and wheat are degraded by various enzymes produced by the *Aspergillus* species. Completed *koji* is then mixed with saline water, which contains 16−18% (w/v) salt to make wet mash or *moromi*. During the *moromi* fermentation for about 6 months, complete degradation of high molecular weight compounds into amino acids, peptides, glucose, and other lower molecular weight compounds is carried out. At the same time, *Saccharomyces* yeasts and lactic acid bacteria generate the unique soy sauce flavor. Finally, raw soy sauce, which is squeezed from the finished *moromi*, is pasteurized so as to add heated aroma by accelerating the Maillard reaction. Thus, the diversity of components contained in the fermented soy sauce is derived from differences in the conditions for heat treatments of raw materials, microorganisms grown in both *koji* and *moromi* stages, and pasteurization conditions. Furthermore, various minor components originally contained in soybeans, such as nicotianamine (Kinoshita *et al*., 1993) and isoflavones, *i.e.*, daidzein and genistein, were also identified in soy sauce (Havsteen, 1983; Wang *et al*., 1990; Coward *et al*., 1993). Recently, many desirable biological activities, such as antifungal (Naim *et al*., 1974), antioxidative (Naim *et al.*, 1976), and estrogenic (Murphy, 1982) activities, were found in various flavonoids which are mainly contained in soybean foods.

* Author to whom correspondence should be addressed (telephone +81 471 23 5590; fax +81 471 23 5550; e-mail QZS01157@niftyserve.or.jp).

Since the late 1960s, multivariate pattern recognition techniques have widely been applied to instrumental data sets of various foods to objectively classify food and beverage samples (Aishima and Nakai, 1991). Objective differentiation of soy sauces produced in different manufacturing conditions has been needed for certificating the authenticity of products. Although gas chromatographic (GC) data of volatile components in soy sauce were studied in detail by using multivariate techniques (Aishima, 1983), pattern recognition analysis for nonvolatile components in soy sauce has not been performed yet. Comparing UV detection in HPLC with flame ionization detection (FID) in GC, FID has many advantages over UV detection mainly because of its lower detection limit and nonspecific response for organic compounds. However, HPLC analysis has advantages over GC analysis, such as higher precision and applicability for nonvolatile components with direct injection of liquid samples. Therefore, in this research, pattern recognition analysis of HPLC profiles in soy sauce was attempted to confirm whether various brands of fermented soy sauce could be differentiated on the basis of their nonvolatile components. Furthermore, elucidation of chemical structures of peak components that played key roles for classifying HPLC profiles according to brands was attempted to understand quality differences of soy sauces in terms of chemical components.

MATERIALS AND METHODS

Materials. Several types of soy sauce are manufactured and distributed in Japan (Fukushima, 1985). Two types of genuine fermented soy sauce were collected on the local market in Japan because of their wider popularity; 51 samples in 8 brands (A−H) of deep-colored, *i.e.*, standard soy sauce (*koikuchi shoyu*), and 37 samples in 6 brands (I−N) of thin-colored soy sauce (*usukuchi shoyu*), which is mainly used as a seasoning for cooking vegetables and/or meats in the western part of Japan.

HPLC Analysis. HPLC analyses were carried out using a Shimadzu liquid chromatograph system with an SIL-10A autoinjector, LC-10AD pumps, and an SPD-10A detector (Shimadzu Corp., Kyoto, Japan). Ten microliters of each soy

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sauce sample was directly injected into a Wakosil-II 5C18 HG column (4.6 mm i.d. × 250 mm, Wako Pure Chemicals Industries, Tokyo, Japan) fitted with a precolumn (4.6 mm i.d. × 30 mm) packed with the same material. The flow rate and column temperature were 0.8 mL/min and 15 °C, respectively, and monitored at 280 nm. The linear gradient chromatography was carried out by using two solvents such as 0.05% trifluoroacetic acid (TFA) in water (I) and 90% acetonitrile containing 0.05% TFA (II). Solvent I was kept at 100% for 20 min, and then solvent II was increased from 0 to 25% for 270 min and then further increased from 25 to 50% for 50 min. After each analysis, the column was washed with tetrahydrofuran. Chromatographic data were accumulated by a Labchart 180 data processor (System Instruments Co., Ltd., Tokyo, Japan).

Pattern Recognition. HPLC data sets of deep-colored and thin-colored soy sauce were separately analyzed by pattern recognition techniques to extract information on brand differentiation. Areas of major 50 peaks were used as variables. Pattern recognition analyses were performed by SPSS for Windows ver. 6.1 (SPSS Inc., Chicago, IL), Unscrambler ver. 5.3 (CAMO AS, Trondheim, Norway), and the GALDA program (Leardi *et al.*, 1992) on a DTK FEAT-5030 486 PC system (DTK, Taipei, Taiwan). In unsupervised pattern recognition, cluster analysis was performed to classify HPLC profiles on the basis of their multidimensional Euclidean distances and the Ward method to investigate mutual relationships among samples using SPSS. In supervised pattern recognition, stepwise linear discriminant analysis (LDA) was performed by SPSS. Next, the most discriminatory subsets of HPLC peaks were selected by the genetic algorithm (GA; Forrest, 1993) using LDA. Further, soft independent modeling of class analogy (SIMCA) was performed using Unscrambler.

Isolation of Peak Components. For elucidating chemical structures of peak components which showed significance for differentiating HPLC profiles in pattern recognition, peak components were isolated and purified through the following steps. Three liters of soy sauce was extracted three times with 1.5 L of ethyl acetate at room temperature for 3 min. After the pH was adjusted to 2.0 with hydrochloric acid, the resulting aqueous layer was further extracted three times with 1.5 L of ethyl acetate for 3 min. The upper layer was collected and concentrated to approximately 13 mL by a rotary evaporator. Amounts of 330 μL of the concentrate were repeatedly injected into preparative HPLC equipped with a Wakosil-II 5C18 HG column (10 mm i.d. × 50 mm + 10 mm i.d. × 300 mm) to separate peak components. The collected fractions containing objective peak components were concentrated and redissolved with 1 M acetic acid in water for further purification with Bio-Gel P2 column (20 mm i.d. × 380 mm, Bio-Rad Laboratories, Cambridge, MA) chromatography eluted with 1 M acetic acid to remove higher molecular contaminants. The UV spectrum of each component was used as indicator in the isolation and purification process by monitoring with an SPD-M10A photodiode array detector (Shimadzu Corp.). Further confirmation of purity was performed with TLC analysis using precoated silica gel 60 plates (Merck & Co., Inc., Rahway, NJ) with a 2-propanol/25% NH_4OH/water (9:1:2) solvent system at a room temperature and detection with sulfuric acid.

Instrumental Analysis. Melting points of purified components were measured by a Yanagimoto micro-melting point apparatus (Yanagimoto Co., Kyoto, Japan). UV spectra of their water solutions were measured by a 557 double-wavelength double-beam spectrometer (Hitachi Ltd., Tokyo, Japan). IR spectra of their KBr disks were obtained with an FT/IR-7300 (Jasco Corp., Tokyo, Japan). NMR spectra of components dissolved in DMSO-d_6 were obtained with a JNM-LA400 (JEOL Ltd., Tokyo, Japan). Their mass spectra were obtained by an FRIT-FAB JMS-AX2000 LC/MS system (JEOL Ltd.).

RESULTS

HPLC Analysis. More than 100 peaks were commonly observed in every HPLC profile as shown in a typical HPLC profile of deep-colored soy sauce (Figure 1). The coefficients of variations (CV) in the 50 major peaks calculated from 8 consecutively repeated HPLC analyses for the same sample deviated from 1 to 27%, but those in most peaks were <10%. Although most peaks were commonly found in all samples, their peak areas considerably differed from brand to brand.

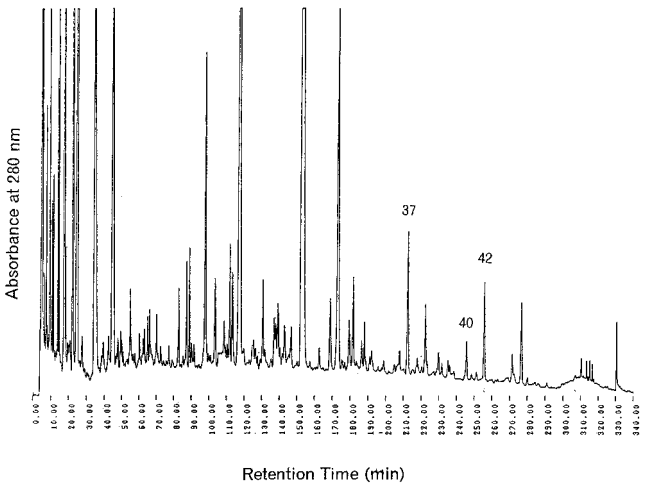

Figure 1. Typical RP-HPLC profiles of deep-colored soy sauce.

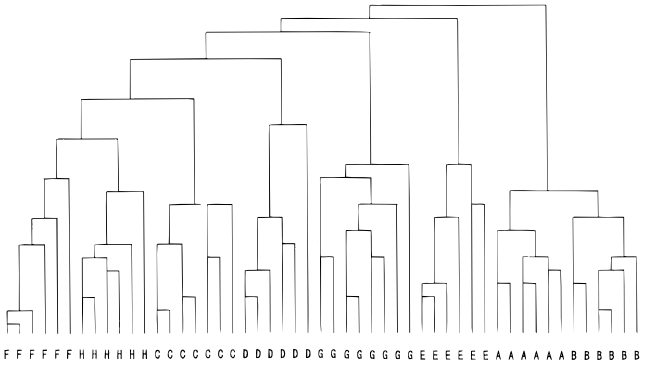

Figure 2. Clustering of HPLC profiles of deep-colored soy sauce, brands A−H.

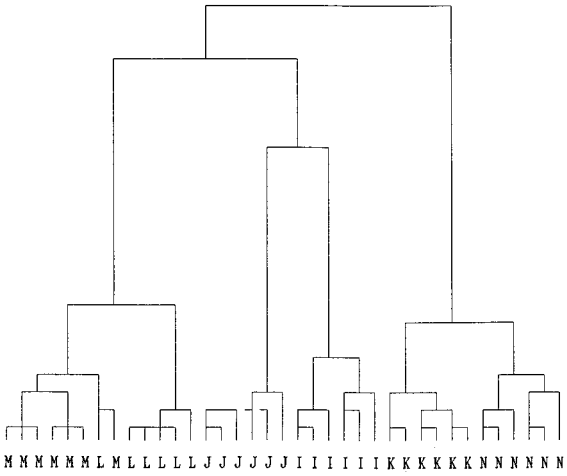

Figure 3. Clustering of HPLC profiles of thin-colored soy sauce, brands I−N.

Pattern Recognition. All HPLC profiles of deep-colored and thin-colored soy sauce were classified according to their brands by cluster analysis (Figures 2 and 3). These neat clusterings indicated that significant differences in whole composition patterns inherently existed among different brands of soy sauces because the clustering was simply performed on the basis of their multidimensional distances without using any

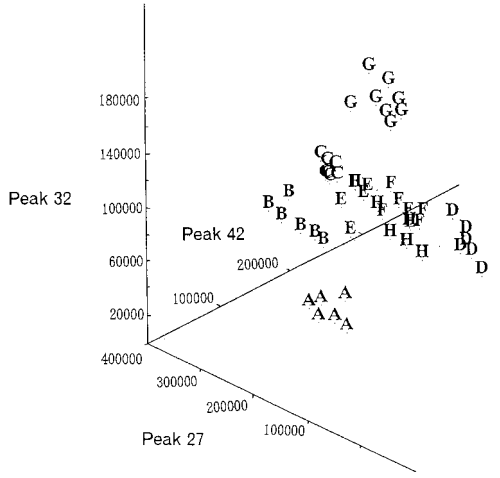

Figure 4. Three-dimensional scatter plot of deep-colored soy sauce based on peaks selected by LDA.

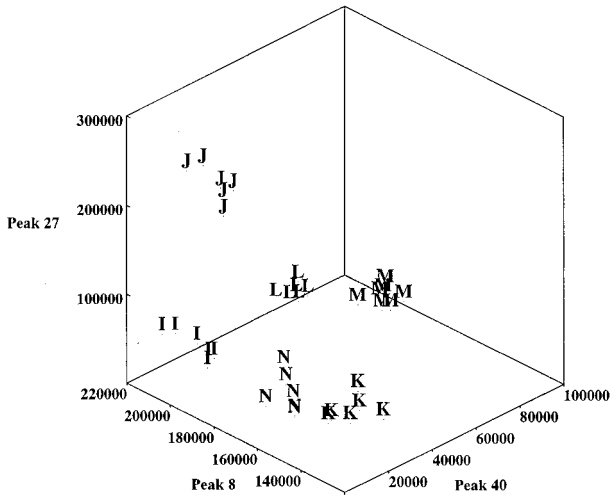

Figure 5. Three-dimensional scatter plot of thin-colored soy sauce based on peaks selected by GALDA.

Table 1. Discirnmination Power of Peaks in SIMCA

peak	mean	SD	peak	mean	SD
9	13.0	6.61	37	23.2	13.30
28	11.0	7.08	40	13.1	7.32
31	10.7	8.88	42	17.9	11.10

criterion for the sample classification. Next, the HPLC data set of deep-colored soy sauce was analyzed by stepwise LDA. The three-dimensional scatter plot based on peaks 27, 32, and 42 selected by LDA is shown in Figure 4. All samples were clearly separated into eight groups, each corresponding to brands A−H. However, the GALDA was applied to the HPLC data of thin-colored soy sauce because the correctly classified ratio based on peaks 27, 31, and 40 selected by stepwise LDA reached only 86.5%. The three-dimensional scatter plot based on peaks 8, 27, and 40 selected by GALDA showed six clearly separated groups corresponding to brands I−N (Figure 5). The discrimination power of peaks, which indicates the contribution of each peak for discriminating two corresponding brands (Sharaf *et al.*, 1986), was obtained from SIMCA analysis applied to the HPLC data set of thin-colored soy sauce (Table 1). Three peaks, 37, 40, and 42, were indicated as the most effective for differentiating six brands of thin-colored soy sauce since their means and standard deviations (SD) of discrimination power were considerably higher than those of other peaks.

Isolation of Three Key Components. Three peaks, 37, 40, and 42, designated A, B, and C, respectively, hereafter, were selected as the most contributory to brand differentiation. Therefore, compounds **A**, **B**, and **C** were isolated and purified according to the aforementioned procedure (Figure 6). From isolation and purification steps, 33.1 mg of **A**, 24.6 mg of **B**, and 5.0 mg of **C** were obtained. Each compound showed single peaks in HPLC analysis and single spots in TLC analysis.

Instrumental Data of Compound A. The melting point of the off-white amorphous powder was 212−218 °C (dec), and UV_{max} (log ϵ_{max}) in water were at 248 nm (4.50) and 302 nm (4.15). The bathochromic UV spectra (Mabry *et al.*, 1970) were as follows: UV_{max} (nm) in MeOH, 233sh, 249, 259, 297sh; + MeONa, 248, 277, 335sh; + $AlCl_3$, 250sh, 262, 296sh; + $AlCl_3$−HCl, 250sh, 261, 296sh; + NaOAc, 250sh, 260sh, 297sh; + NaOAc−H_3BO_3, 250sh, 260sh, 297sh. The ^{1}H-NMR and ^{13}C-NMR data are shown in Table 2. High-resolution FAB-MS: found, *m/z* 387.0722 $[M + H]^+$; calcd for $C_{19}H_{14}O_9$. Negative FAB-MS: *m/z* 385 $[M - H]^-$ and 253 (Figure 7A). IR_{max} (cm^{-1}): 3420 (br, OH), 1620, 1520, 1450, 1250, 1200, 1110, and 840.

Instrumental Data of Compound B. The melting point of the pale yellow amorphous powder was 138−144 °C (dec), and UV_{max} (log ϵ_{max}) in water was at 260 nm (4.63). The bathochromic UV spectra were as follows: UV_{max} (nm) in MeOH, 261, 324sh; + MeONa, 270; + $AlCl_3$, 266; + $AlCl_3$−HCl, 267, 379; + NaOAc, 262, 322sh; + NaOAc−H_3BO_3, 262, 322sh. The ^{1}H-NMR and ^{13}C-NMR data are shown in Table 2. High-resolution FAB-MS: found, *m/z* 403.7061 $[M + H]^+$; calcd for $C_{19}H_{14}O_{10}$. Negative FAB-MS: *m/z* 401 $[M - H]^-$ and 269 (Figure 7B). IR_{max} (cm^{-1}): 3450 (br, OH), 650, 1620, 1580, 1520, 1360, 1250, and 840.

Instrumental Data of Compound C. The melting point of the pale yellow amorphous powder was 209−212 °C (dec), and UV_{max} (log ϵ_{max}) in water was at 266 nm (4.50). The bathochromic UV spectra were as follows: UV_{max} (nm) MeOH, 266, 307sh, 356; + MeONa, 278, 338; + $AlCl_3$, 275, 312sh, 366; + $AlCl_3$−HCl, 278, 311sh; + NaOAc, 267sh, 392; + NaOAc−H_3BO_3, 267. The ^{1}H-NMR and ^{13}C-NMR data are shown in Table 2. High-resolution FAB-MS: found, *m/z* 419.0639 $[M + H]^+$; calcd for $C_{19}H_{14}O_{11}$. Negative FAB-MS: *m/z* 417 $[M - H]^-$ and 285 (Figure 7C). IR_{max} (cm^{-1}): 3450 (br, OH), 1650, 1640, 1620, 1540, 1520, 1510, 1460, 1420, 1220, 1100, 1050, and 840.

Structure Elucidation of the Three Compounds. Sharp singlet signals at δ 8.33, 8.35, and 8.50 in the ^{1}H-NMR spectra of **A**, **B**, and **C**, respectively, were typical for the position 2 protons (H-2) in isoflavone moieties. However, ^{1}H-NMR spectra of three compounds showed no signal assignable to a methoxyl group which is characteristic in glycitein. Concerning NMR spectra of **A** and **B**, all signals were assigned to daidzein and genistein except for signals at δ 4.41 and 5.02 in the ^{1}H-NMR spectra and at δ 72.0, 78.8, 168.4, and 171.0 in the ^{13}C-NMR spectra. Regarding the ^{1}H-NMR spectrum of compound **C**, signals at δ 8.72 and 12.3 were assigned to hydroxy groups attached to the A-ring of isoflavone and the whole spectrum suggested that compound **C** might be a derivative of hydroxygenistein. Therefore, chemical shifts in the ^{1}H-NMR, ^{13}C-NMR, and HSQC spectra suggested that daidzein, genistein, and hydroxygenistein in which another hydroxy group was attached to the A ring were contained as their partial structures. The molecular formulas of com-

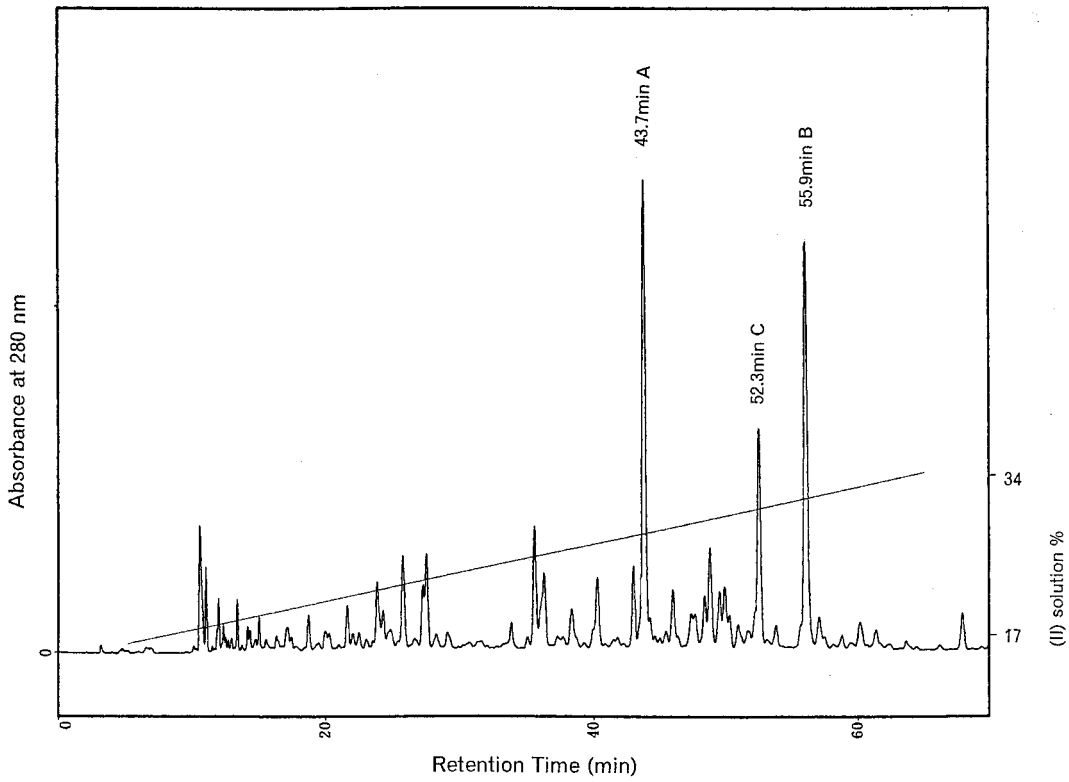

Figure 6. Preparative HPLC chromatogram of ethyl acetate extract.

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds A−C in DMSO-d_6

	A				B				C			
position	δ^{13}C	δ^1H	*J* (Hz)	HMBC	δ^{13}C	δ^1H	*J* (Hz)	HMBC	δ^{13}C	δ^1H	*J* (Hz)	HMBC
2	153.1	8.33 s			154.4	8.35 s			154.3	8.50 s		
3	123.7			H-2, 2′	122.6			H-2, 2′	122.1			H-2, 2′
4 (C=O)	174.6			H-2, 5	180.4			5-OH	180.8			H-2
5	127.0	8.02 d	8.8		161.7			H-6, 5-OH	152.8			H-6, 5-OH
6	115.0	7.07 dd	2.2, 8.8	H-8	98.8	6.35 d	2.2	H-8	98.1	6.42 s		5-OH
7	161.9			H-5, 8, 3″	163.5			H-6, 8, 3″	151.6			H-6, 3″
8	102.1	7.15 d	2.2	H-6	93.6	6.59 d	2.2	H-6	126.9			H-6
9	157.0			H-2, 8	157.3			H-2, 8	145.1			H-2
10	118.1			H-6, 8	105.8			H-6, 8	105.7			H-6, 5-OH
5-OH						12.90 s				12.30 br		
8-OH										8.72 br		
1′	122.3			H-2, 3′	121.0			H-2, 3′	121.1			H-2, 3′
2′	130.0	7.40 d	8.6	H-3′	130.1	7.39 d	8.8	H-3′	130.1	7.39 d	8.8	
3′	115.0	6.81 d	8.7	H-2′	115.1	6.83 d	8.8	H-2′	115.0	6.81 d	8.8	H-2′
4′	157.2			H-2′, 3′	157.5			H-2′, 3′	157.4			H-2′, 3′
4′-OH		9.45 br				9.55 br				9.55 br		
1″ (C=O)	168.4[a]			H-2″, 3″	168.3[a]			H-2″, 3″	168.6[a]			H-2″, 3″
2″	72.0	4.41 d	2.2	H-3″	71.0	4.56 d	2.7	H-3″	71.1	4.63 d	2.9	H-3″
3″	78.8	5.02 d	2.2	H-2″	78.7	5.22 d	2.7	H-2″	79.7	5.32 d	2.9	H-2″
4″ (C=O)	171.0[a]			H-2″, 3″	171.5[a]			H-2″, 3″	171.0[a]			H-2″, 3″

[a] Assignments for these signals within the same column may be interchanged.

pounds **A**, **B**, and **C** were found to be $C_{19}H_{14}O_9$, $C_{19}H_{14}O_{10}$, and $C_{19}H_{14}O_{11}$ $[M + H]^+$, respectively, by high-resolution FAB-MS, respectively. Negative FAB-MS $[M - H]^-$ fragments, *m*/*z* 253, 269, and 285, were attributable to three isoflavone moieties, such as daidzein, genistein, and hydroxygenistein. Thus, both NMR and FAB-MS spectral data strongly suggested that three purified components were isoflavone derivatives.

Next, the substitution position of the hydroxy group in compound **C** should be decided since there were two possible positions in the A ring, *i.e*, H-6 or H-8. According to the HMBC spectrum shown in Figure 8, the cross-peaks of 5-OH/C-6 and H-6/C-5 indicated that this aglycon was 8- hydroxygenistein, and other cross-peaks supported this assumption as well. If H-6 would be substituted, 5-OH/C-8 and H-8/C-9 correlations should not appear at the positions shown in Figure 8. Thus, the aglycon of compound **C** was decided as 8-hydroxygenistein.

Two proton signals at δ 4.41 and 5.02 and four carbon signals at δ 72.0, 78.7, 168.4, and 171.0 commonly appeared in NMR spectra of **A**−**C**. However, those signals could not be assigned to isoflavone skeletons. From signals at δ 4.41 and 5.02 in the ^{1}H-NMR spectra and at δ 72.0 and 78.8 in the ^{13}C-NMR spectra the presence of two methenyl groups connected to oxygen atoms (>CHO−) in their partial structures was suggested. Furthermore, the presence of two carboxyl groups (>C=O) in their partial structures was indicated from signals at δ 168.4 and 171.0 in the ^{13}C-NMR spectra. According to the aforementioned interpretation of chemical shifts, the entire structures of **A**−**C** were

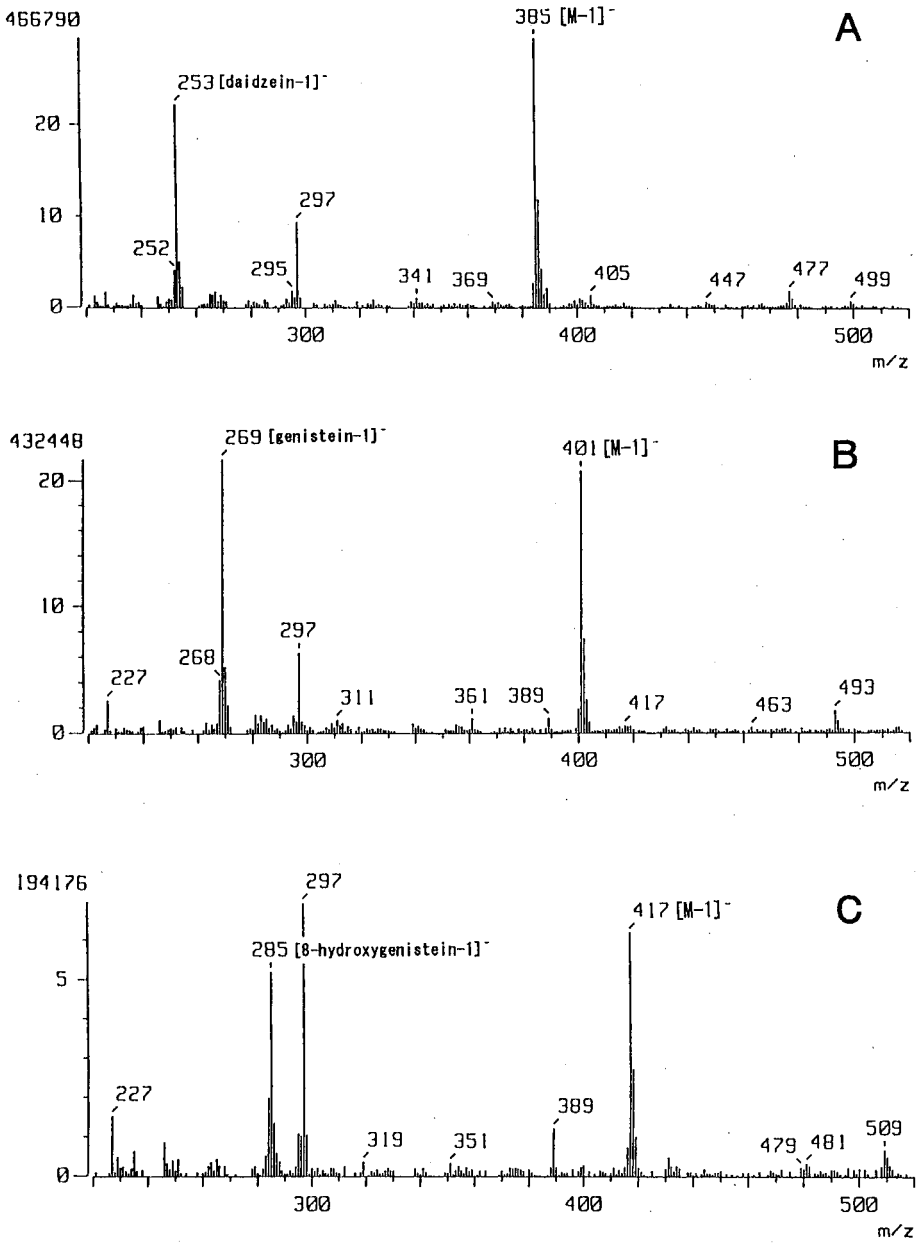

Figure 7. Negative FAB-MS spectra of compounds **A**−**C**.

assumed as 2,3-dihydroxysuccinic acid, or tartaric acid, connected to isoflavone moieties.

Finally, the connectivity between isoflavone moieties and tartaric acid was decided on the basis of the HMBC spectra of **A**−**C**. The cross-peaks of C-7/H-3″ in every HMBC spectrum indicated a three-bond connectivity with ether linkage. The structures of compounds **A**−**C** thus elucidated are shown in Figure 9.

DISCUSSION

Successful pattern recognition results suggested that HPLC analysis is an appropriate method for classifying soy sauces of different brands because all clusters and scatter plots obtained from cluster analysis, LDA, and SIMCA clearly showed differences in profiles of nonvolatile components from brand to brand. The peak selection by GALDA showed especially superior capability over the stepwise LDA. This superiority was derived from the difference in the basic algorithms for peak selection between the two LDA methods. That is, peaks were selected so as to maximize the correct classification ratio by examining their reliability using the cross-validation (Sharaf *et al.*, 1986) in GALDA, but the selection was performed to simply maximize the ratio of within-group variance to between-group variance in the stepwise LDA. The larger ratio of the two variances does not necessarily mean highly correct classification.

Until now, three soy isoflavone aglycons, daidzein, genistein, and glycitein, were found in four isomeric forms in soybeans and soybean foods, and their distribution was quantified in soybeans and soybean foods by Wang and Murphy (1994a,b, 1996). György *et al.* (1964) found 6,7,4′-trihydroxyisoflavone (factor 2) in tempeh, which is a fermented soybean food that originated in Indonesia. However, this is the first report on isoflavone derivatives combined with tartaric acid via ether linkage. To date, compounds connected to tartaric acid with an ester linkage such as caffeoyl esters have been reported (Snook *et al.*, 1994), but no compound combined to tartaric acid with ether linkage has been reported yet. Although most isoflavone aglycons are contained as the form of isoflavone glucosides in soy-

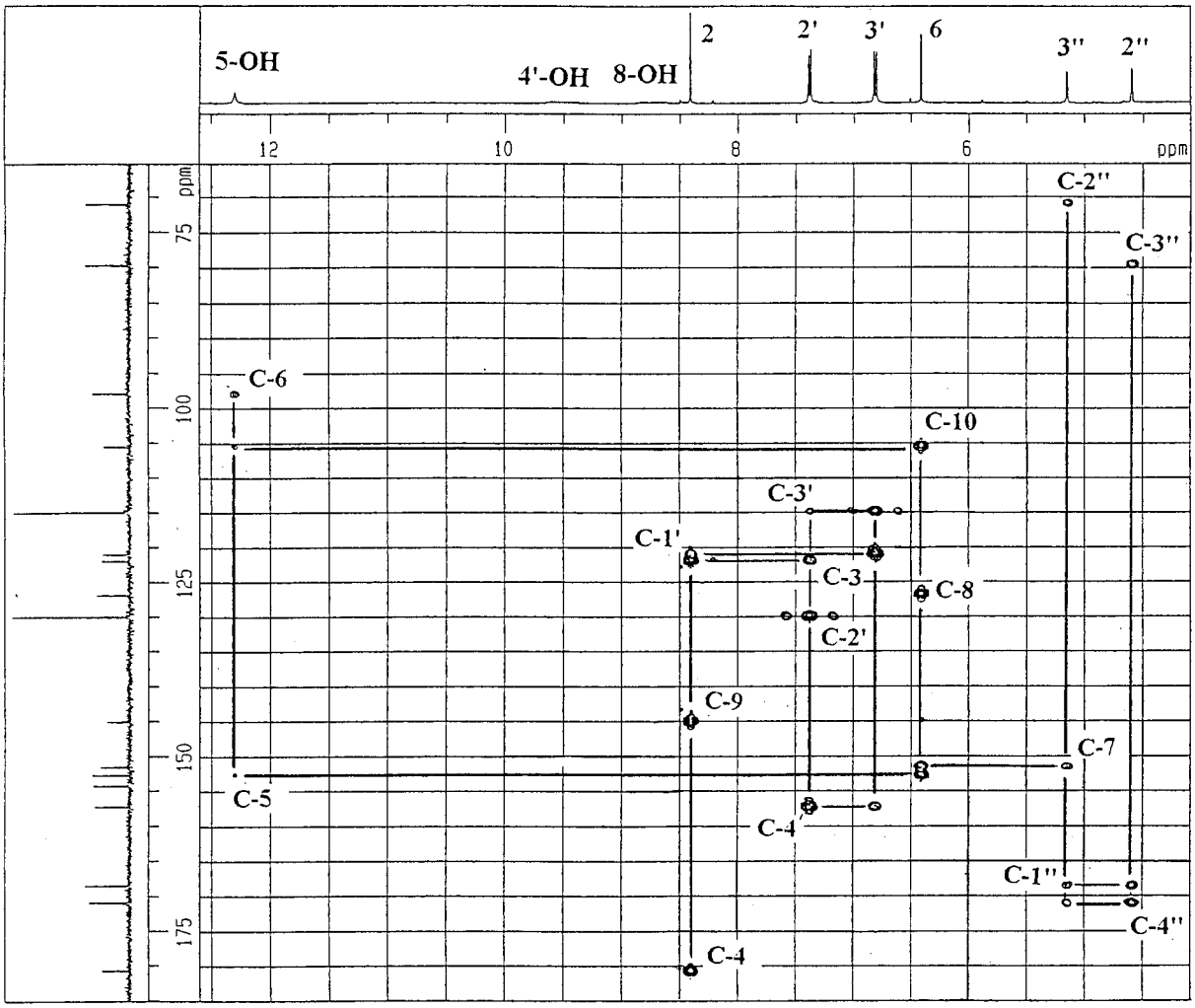

Figure 8. HMBC spectrum of compound **C**.

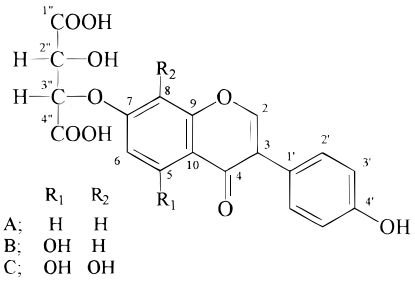

Figure 9. Structures of compounds **A**−**C**.

beans, those aglycons are liberated from sugar parts by enzymic action during fermentation periods of soybean foods (Wang and Murphy, 1996). Similarly, three new isoflavones, **A**, **B**, and **C**, seem to be generated during the soy sauce fermentation. However, these isoflavone derivatives have not been found in any other soybean foods yet.

Although transformation pathways have not been fully understood yet, some isoflavones transformed by various microorganisms have been reported recently (Nakahara *et al.*, 1986; Klaus *et al.*, 1993). Umezawa *et al.* (1975) reported that 8-hydroxygenistein was produced in the culture of *Aspergillus niger*. Their findings suggested two possible pathways for generation of compound **C**, *i.e.*, direct transformation from **B** or via 8-hydroxygenisten which is first transformed from genistein during fermentation. The study of configurations and synthetic pathway of the three new isoflavones is now under way.

ABBREVIATIONS USED

CV, coefficient of variation; FAB-MS, fast atom bombardment mass spectometry; FID, flame ionization detection; i.d., inside diameter; IR, infrared; LDA, linear discriminant analysis; GALDA, linear discriminant analysis using genetic algorithm; GC, gas chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; RP, reversed phase; SD, standard deviation; SIMCA, soft independent modeling of class analogy; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; UV, ultraviolet.

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