

Separation of Flavonoids by Semi-Micro High-Performance Liquid Chromatography with Electrochemical Detection

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A highly sensitive and selective method was developed for the determination of 15 flavonoids by high-performance liquid chromatography with electrochemical detection (HPLC–ECD) using a microbore column. The 15 flavonoids were divided into two groups according to their hydrophobicity and were resolved by two isocratic systems, namely, methanol–water (1:1 and 3:7, v/v) containing 0.5% phosphoric acid. The retention factor (k) of each flavonoid linearly correlated with the logarithmic partition of coefficients between 1-octanol–water ($\log P$) values. The detection limits ($S/N = 3$) of the flavonoids tested were in the range of 2–25 fmol; that is 600 times more sensitive than conventional HPLC with UV detection. The relative standard deviations (RSD, $n = 5$) were less than 5% at 1 pmol for the flavonoids tested. By the present method, only 100 μL of bottled Japanese green tea were enough to determine the flavonoid contents.

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin[1] and in some herbal drugs.[2] The potent biological effects of flavonoids have been described in many in vivo and in vitro studies.[3] These studies point to the potentially beneficial health effects of dietary flavonoids.[4] Recently, it has also been reported that a high intake of flavonoids has been inversely associated with subsequent heart disease in several prospective studies.[5,6]

Research on flavonoids in relation to the human diet has mainly concentrated on flavonols and catechines,[7–9] partly due to early methods enabling sensitive detection of these compounds in biological fluids and foods. However, over 50 flavonoids are common in foods,[7] and it has recently been shown that other subclasses of flavonoids may also be important to human health. These are: citrus flavones, isoflavones found in soy food, and flavanones, since they are presumably absorbed to a higher extent than flavonols[10–12] and are present in some herbal drugs where flavanone content has been reported to be very high.[13] Therefore, detection and separation of flavonoids from each other would be quite valuable.

Although a few analytical methods[14–16] have been developed that can simultaneously determine several subclasses of flavonoids in foods, these methods, when applied to biological fluids, do not meet the necessary sensitivity requirements. An analytical method with lower detection limits, based on high-performance liquid chromatography (HPLC) with fluorometric detection (FL), is limited to the detection of flavonols possessing a 3-OH group.[8] Collectively, these reports support the notion that, if we develop a more sensitive and selective method for the determination of flavonoids, such a method would be quite useful for future flavonoid research.

Because of the beneficial health effects of flavonoid antioxidant activities, many researchers have shown interest in this field. If one wants to develop the above-mentioned useful method, electrochemical detection (ECD) should be an appropriate detection method. ECD is based on the redox reaction on the electrode, leading to the achievement of high selectivity. Because the redox reaction of flavonoids may often relate to their biological functions, ECD may help predict the antioxidant functions of flavonoids. In fact, we have previously shown that the oxidation potential of flavonoids, obtained by cyclic voltammetry and flow-through column electrolysis, was associated with their antioxidant activities.[17] In addition, another merit of ECD is its ability to detect even unknown antioxidative flavonoids in foods, biological samples, or Chinese herbal medicines. As a separation method, HPLC would be effective. If we use a reversed-phase system, the retention should largely associate with the chemical structure and the hydrophobicity of flavonoids. Information regarding the retention will also help in the prediction of other biological functions of flavonoids, such as membrane permeability and absorption through the gastrointestinal tract. From the above observations, we have been encouraged to develop a more sensitive and selective analytical method for flavonoids by HPLC–ECD.

Although liquid chromatography with mass spectrometry detection (LC–MS) is gaining popularity as an analytical method, this detection method cannot give any functional information including the antioxidant activity of flavonoids. So, we consider both HPLC–ECD and LC–MS to be in practical use where both methods act in ways that are complementary to each other. In our previous work on the development of a method for the determination of catechins,[18] HPLC–ECD using a microbore column was proven to be 100 times more sensitive when compared to a conventional column. Thus, semi-micro HPLC–ECD may be a promising method for a sensitive and selective determination of flavonoids. In this paper, we have selected 15 flavonoids (seven flavonols, four flavones, two flavanones, and two isoflavones) (Fig. 1) on the basis of their availability as

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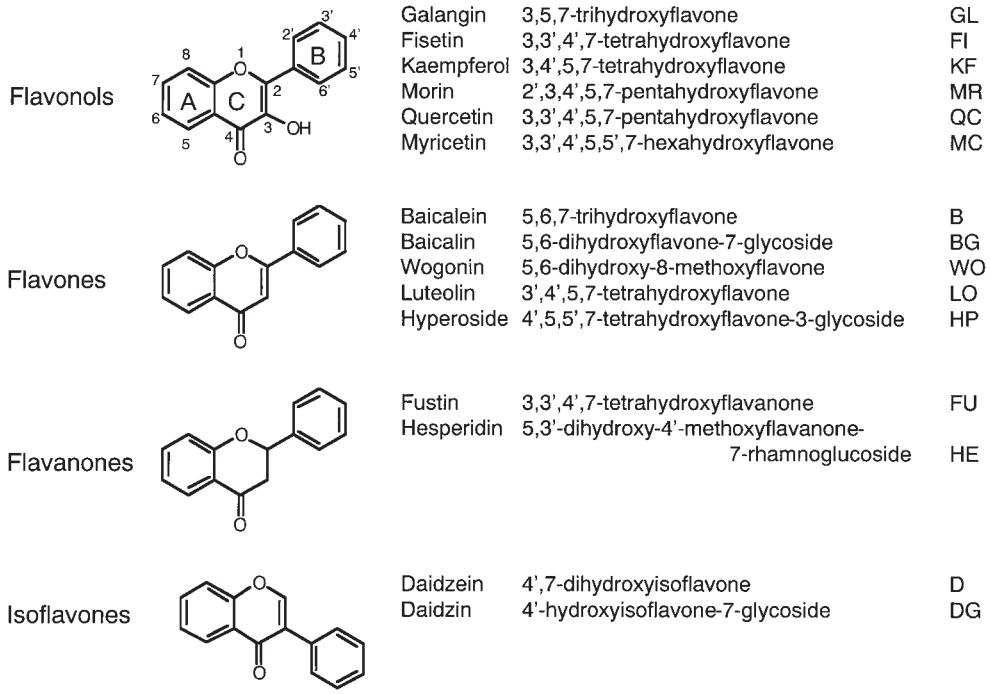

Fig. 1. Structures of the flavonoids examined.

standard compounds, and have developed a highly sensitive and selective method for the determination of those flavonoids with HPLC–ECD using a microbore column.

Because the beneficial health effects of Japanese green tea, often attributed to flavonoids as described above, have become a recent topic of interest, we have decided to use commercial bottled Japanese green tea for this study to examine the applicability of the present method. To explore the mechanisms of the effect of flavonoids in Japanese green tea, an appropriate analytical method is necessary. However, the analysis of Japanese green tea is generally difficult because flavonoid content as well as other substances vary with every extraction, and depend on variables such as: the ratio of tea leaves to hot water, the time of incubation, and the temperature of the hot water used. Moreover, in experiments with small animals such as mice, we get a very small amount of blood or urine samples. So, a highly sensitive and selective analytical method is required. By applying the present method, we demonstrated that only 100 μL of bottled Japanese green tea was enough to determine flavonoid content.

Experimental

Chemicals. Quercetin dihydrate (QC), baicalein (B), baicalin (>99.0%) (BG), daidzein (>97%) (D), daidzin (>99%) (DG), wogonin (>98%) (WO), fisetin (FI), luteolin (>90%) (LO), galangin (GL), and kaempferol (95%) (KF) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Myricetin (MC), hesperidin (80%) (HE), and morin (MR) were purchased from Sigma Chemical Co. (St. Louis, MO). Fustin (FU) and hyperoside (HP) were from Funakoshi Co. (Tokyo, Japan). Methanol (MeOH) and other reagents were of HPLC grade (Wako Pure Chemicals). Deionized and distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification. Japanese green tea (Kirin Co. Ltd, Tokyo, Japan) was purchased from commercial sources.

Apparatus and HPLC–ECD Conditions. The HPLC–ECD equipment with a microbore column comprised of an LC-27A vacuum degasser (BAS, Tokyo, Japan), an LC-100 pump (BAS), a 7125 injector fitted with a 5 μL injection loop (Reodyne, Cotati, CA, USA), a CAPCELL PAK C18 UG 120 microbore ODS column (150 mm × 1.0 mm i.d., 3 μm, Shiseido, Tokyo, Japan), an FT-1 column oven (BAS), and an LC-4C electrochemical detector (BAS). The commercially available electrochemical cell (Radial flow cell, BAS) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. A 5 μL sample solution or standard flavonoid solution was injected into the microbore ODS column maintained at 40 °C. Deaerated MeOH–water (1:1, 4:6, or 3:7, v/v) containing 0.5% phosphoric acid served as the mobile phase, at a flow rate of 25 μL/min. The detection potential for flavonoids monitoring was set at +0.8 V vs Ag/AgCl. Each flavonoid concentration in the sample solution was determined based on current peak height.

Sample Preparations. Bottled Japanese green tea (100 μL) was mixed with 30 μL of 2.5 μM of B in MeOH–water (4:6, v/v) containing 0.5% phosphoric acid as an internal standard and 120 μL of MeOH–water (4:6, v/v) containing 0.5% phosphoric acid. The mixture was sonicated for 3 min, then added to 50 μL of 6 M HCl and incubated for 1 h at 90 °C to hydrolyze the conjugated flavonoid forms. The hydrolyzed mixture was filtered through a 0.45 μm filter. Ten μL of the mixture was diluted with 990 μL of MeOH–water (4:6, v/v) containing 0.5% phosphoric acid, and 5 μL of the diluted mixture was applied to the semi-micro HPLC–ECD.

Results and Discussion

Figure 2 shows a typical chromatogram of the separation achieved using MeOH–water (1:1, v/v) containing 0.5% phosphoric acid as mobile phase (Elution System I) on a microbore column. The signal currents were detected at +0.8 V vs Ag/AgCl. As shown in Fig. 2, QC, LO, KF, B, WO, and GL were

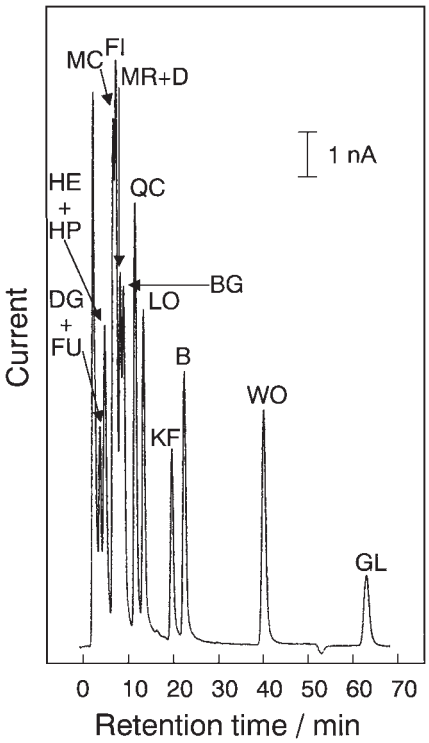

Fig. 2. Chromatographic separation of 15 flavonoids with MeOH–water (1:1) containing 0.5% phosphoric acid as mobile phase (Elution System I). Other HPLC conditions as in the "Experimental" section.

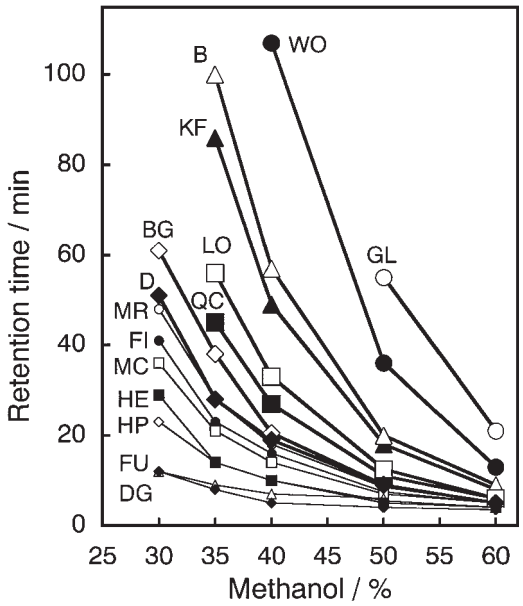

Fig. 3. Effects of MeOH in mobile phase on elution of flavonoids. Mobile phase, MeOH–water containing 0.5% phosphoric acid. Other HPLC conditions as in the "Experimental" section.

sufficiently resolved. However, some peaks, i.e., FU and DG, HP and HE, MR and D overlapped each other. Insufficient peak separations were also observed between MC, FI, and BG. To improve the separation, the elution of an individual flavonoid (Fig. 1) was studied by isocratic elution with MeOH–water containing 0.5% of phosphoric acid as a mobile phase. Figure 3 shows the correlation between retention time and MeOH concentration in mobile phase. The approximate elution order is as follows: FU, DG, HP, HE, MC, FI, MR, D, BG, QC, LO, KF, B, WO, and GL (Fig. 3). The elution sequence of the individual flavonoids appears to depend on the increased or decreased polarity of the compounds and also on the extent of hydrogen bonding.[19] These separations were conducted on the reversed-phase column; therefore, as expected, flavonoid glycosylation had a pronounced effect on the retention time of the analyte by reducing the retention for each of the classes examined (compare DG and D, BG and B) and the most polar flavonoids (HP, HE) eluted early. Interestingly, the effect on retention time was fairly uniform regardless of the position of glycosylation. Analogous arguments regarding the effect of hydroxy groups on the retention times of these flavonoids have also been made.[19–21] Hydroxy groups at positions other than three and five on rings A or C rendered a flavonoid more hydrophilic. This is supported by the elution orders of MC, MR, QC, KF, and GL. Hydroxy groups at positions three and five on rings A and C increased the retention time. This is evidenced by the elution orders of QC and FI. This can best be explained by the formation of internal hydrogen bonding between a carbonyl group at position four on ring C and the hydroxy group at position five in ring A of these compounds, respectively. Saturation of ring C decreased the retention time. This became evident when comparing FU and FI. This is probably due to the interruption of conjugation that affects the hydrogen-accepting or -donating abilities of all the hydroxy groups. Therefore, the larger the number of hydroxy groups, the more pronounced is the decrease in retention time.

As shown in Fig. 2, the separation of the 15 flavonoids was difficult with one isocratic run. Based on the data shown in Fig. 3, the mobile phase, MeOH–water (3:7, v/v) containing 0.5% phosphoric acid (Elution System II) was tried for the separation of FU, DG, HP, HE, MC, FI, MR, D, and BG. As shown in Fig. 4, all the flavonoids tested, except FU and DG, were completely separated by Elution System II. Thus, when the present method was applied to real samples, Elution System I would be recommended as a first choice for the determination of QC, LO, KF, B, WO, and GL, and to roughly know the contents of other flavonoids. If it is necessary to perform further analyses of other flavonoids, one can use Elution System II to determine the remaining FU, DG, HP, HE, MC, FI, MR, D, and BG. So, we believe that the two isocratic systems (Elution Systems I and II) were sufficient for the determination of the 15 flavonoids tested in this paper.

As an additional option, MeOH–water (4:6) containing 0.5% phosphoric acid (Elution System III) was also examined. To know the oxidation potential of each flavonoid by Elution System III, hydrodynamic voltammograms of the flavonoids (1 pmol of each) were measured. The oxidation current of most of the flavonoids appeared around at +0.4 V vs Ag/AgCl, and the current increased when the applied potential became more positive, while the oxidation current of DG, HE, or D appeared around at +0.8 V vs Ag/AgCl. So, when we set an applied potential at +0.6 V vs Ag/AgCl, DG, HE, and D were not detected, whereas other flavonoids including FU, HP, MC, FI, MR, BG, QC, LO, KF, and B were selectively detected. As

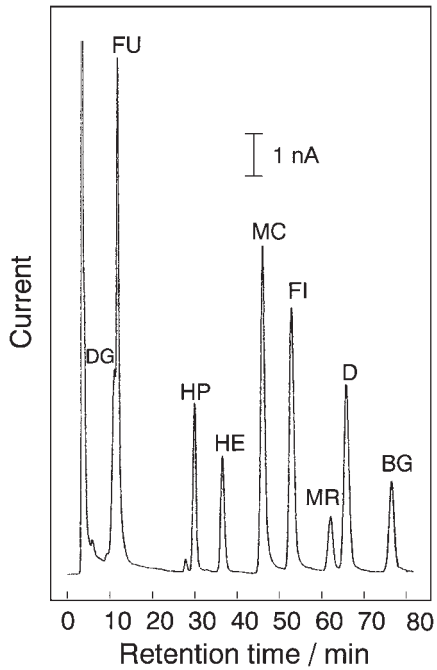

Fig. 4. Chromatographic separation of 9 flavonoids with MeOH–water (3:7) containing 0.5% phosphoric acid as mobile phase (Elution System II). Other HPLC conditions as in the "Experimental" section.

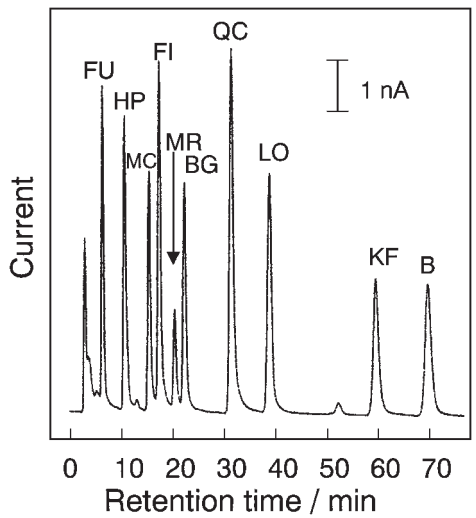

Fig. 5. Chromatographic separation of 10 flavonoids with MeOH–water (4:6) containing 0.5% phosphoric acid as mobile phase (Elution System III). Applied potential, +0.6 V vs Ag/AgCl. Other HPLC conditions as in the "Experimental" section.

shown in Fig. 5, those 10 flavonoids were separated and detected on a chromatogram in a single HPLC run. Therefore, to see whether the targeted flavonoids are included in those 10 flavonoids, examination with Elution System III on the applied potential at +0.6 V vs Ag/AgCl would be a useful option to examine.

The logarithmic partition of coefficients between 1-octanol–water (log *P*) values of the flavonoids, which is widely used as a hydrophobicity scale, was calculated by Prolog P 5.1 software,[22–24] and plotted against the retention factor (*k*). As shown in Fig. 6C, the slope of Elution System I was found to be approximately linear with the coefficient of correlation, $r = 0.8707$. Similar results were obtained with Elution System III (Fig. 6B). The *k* values of the glycosides (HP, BG, HE, and DG) that were well resolved in Elution System II (Fig. 4) did not show any linearity to the log *P* value. Therefore, the flavonoid *r* values in Elution System II were less than 0.6. Instead of Elution System II, we plotted the *k* values of flavonoids in MeOH–water (35:65, v/v) containing 0.5% phosphoric acid against the log *P* value (Fig. 6A), indicating a good correlation again ($r = 0.7897$). From these results, we have concluded that the retention factor of the flavonoid tested was linearly related to the log *P* value, indicating that the hydrophobicity of the flavonoid is a critical factor for retention. Moreover, it seems to be therefore possible to estimate the retention factor if we know the structure of flavonoids before the HPLC run. However, the *k* values of several flavonoids including FI and MC did not show a good correlation to the log *P* values (Fig. 6). For

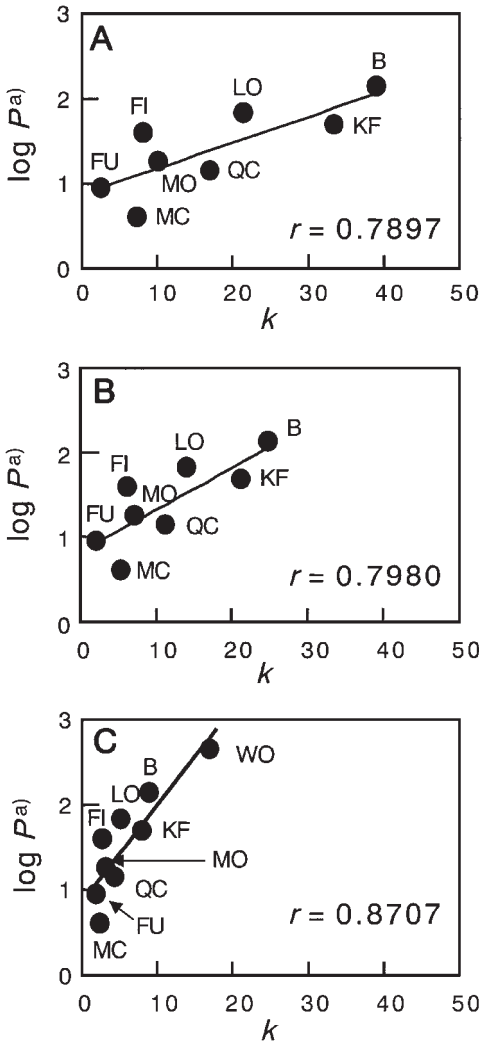

Fig. 6. Relationship of the retention factor (*k*) and the calculated log *P* values of flavonoids. Methanol ratio in mobile phase was (A) 35, (B) 40 (Elution System III), and (C) 50% (Elution System I), respectively. [a)] log *P* values were calculated by the Prolog P 5.1 software.[22–24]

Table 1. Detection Limit of Flavonoids by Several Methods

Method	Detection limit/pmol	Flavonoid	Column		Injection volume/μL	Reference
			i.d./mm	Length/mm		
Semimicro HPLC–ECD	0.002	LO	1.0	150	5	Present method
	0.002	QC	1.0	150	5	Present method
	0.002	MC	1.0	150	5	Present method
	0.002	KF	1.0	150	5	Present method
HPLC–ECD	0.06	QC	4.0	250	30	26
HPLC–UV	1.3	MC	4.6	100	10	25
HPLC–UV	9.3	QC	4.0	250	20	15
HPLC–FL	0.01	QC	4.6	150	20	8
	0.028	MC	4.6	150	20	8
	0.004	KF	4.6	150	20	8
LC–MS–MS	0.007	QC	4.6	250	20	27

The detection limits were expressed by the absolute amounts of the flavonoid (pmol) rather than the concentration.

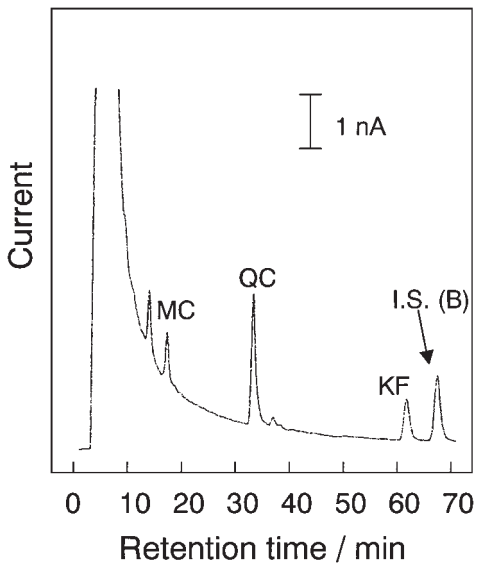

Fig. 7. Chromatogram of bottled Japanese green tea by the present method. The HPLC conditions were same as in Fig. 5.

FI, this compound does not have a hydroxy group at position five, so FI does not have the apparent conjugated structure formed by a carbonyl group at position four and a hydroxy group at position five in other flavonols, probably leading to a different chromatographic behavior. For MC, six hydroxy groups may form an unexpected stereochemical structure that affects the hydrophobicity calculated by the Prolog P 5.1 software. When put together, although the k values of the most flavonoids associate well with the calculated log P values, there are several exceptions.

To evaluate the method described in this paper, we compared the detection limits of the indicated flavonoids in the present method with those in other methods reported (Table 1). The detection limits of LO, QC, MC, and KF by the present method were 0.002 pmol ($S/N = 3$). For MC, the detection limit was 600 times more sensitive than that of the HPLC–UV using a conventional column.[25] For QC, the detection limit was 1000 times more sensitive than that of the HPLC–UV.[15] In addition, the detection limit was 30 times greater than that of the HPLC–ECD using a conventional column.[26] Moreover, the detection limit was almost comparable and slightly higher than to that of LC–MS–MS.[27] Thus, HPLC–ECD with the microbore column is highly sensitive, indicating that a small amount of samples is enough when applied to real samples. Although the sensitivity of KF, QC, and MC (0.004, 0.01, and 0.028 pmol, respectively) by HPLC–FL is similar to that for the present method, it suffers by limitations in the number of structurally different flavonoids.[8] The linear relations of calibration curves were observed in the range 0.01–1 pmol ($r > 0.999$) for all flavonoids tested, except DG (0.025–1). Relative standard deviations (RSD, $n = 5$) were in the range 1.4–4.9% at 0.1 pmol flavonoids tested.

Table 2. Flavonoids Contents in the Bottled Japanese Green Tea

Flavonoids	Content ($n = 5$)	
	Concentration/μmol L^{-1}	RSD/%
MC	2.8	2.1
QC	6.4	2.1
KF	5.3	2.5

Only 100 μL of the bottled Japanese green tea was used. The HPLC conditions were same as in Fig. 5.

Finally, we determined the flavonoid contents in bottled Japanese green tea in order to test the applicability of the present method to real samples. As mentioned above, it is necessary to develop an analytical method for the determination of flavonoids in a small amount of Japanese green tea. Figure 7 shows a representative chromatogram of flavonoids of bottled Japanese green tea. Because our preliminary experiment showed that the bottled Japanese green tea did not contain detectable levels of B, the internal standard method using B was utilized to determine the flavonoid content in the bottled Japanese green tea (Table 2). RSD ($n = 5$) in three flavonoids was less than 2.5%, indicating the present method provided quite accurate measurements of flavonoids in quite small amounts (<100 μL) of bottled Japanese green tea.

Conclusions

The proposed method was developed for the separation of 15 flavonoids with two elution systems. Moreover, the following

points should be taken into consideration. First, the microbore column with ECD increases sensitivity through a decrease in band broadening that results in sharper peaks. Therefore, the present HPLC–ECD with a microbore column became a more sensitive method for determining flavonoids when compared to other known methods. Furthermore, the low flow rate (25 μL/min) is also a considerable advantage to the environment because of less consumption of the organic solvent present in the mobile phase. Second, if we meet unknown flavonoids, we can expect their log *P* values based on their retention factors. Alternatively, if we know the structure of flavonoids before the experiment, we can calculate the log *P* value, thereby estimating the retention factor. Thus, due to good sensitivity, selectivity, and separation ability, the proposed method is likely to be useful for the determination of the composition ratio of flavonoids in real samples.

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