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Enantioseparation of Physiologically Active Some Flavonoids by Liquid Chromatography-Electrospray-Tandem Mass Spectrometry Based on Noncovalent Interactions with β-Cyclodextrin

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Flavonoids are a group of polyphenolic compounds that include a common benzo-γ-pyrone structure and commonly found in fruits, vegetables, tea and red wines.[1,2] Flavonoids are classed as flanones, flavonols, flavanones, isoflavanones, anthocyanidins, catechins and so on. Among the flavonoids, flavanones and flavan-3-ols have a structural characteristic known as chirality. The molecules are also receiving much attention due to their physiological and pharmacological importance.[3] Furthermore, it has been reported that they are beneficial to human health based on their anticarcinogenic, antiallergic, or antiviral activities.[4-6] Thus, stereospecific assay methods for their medicinal and phamacometric studies are needed.

A variety of methods of analysis and separation of chiral flavonoids have been extensively carried out for over 20 years.[7] Mainly, chiral flavonoids have been resolved by chromatographic methods such as high-performance liquid chromatography/ultraviolet detector (HPLC/UV)[8-10] and capillary electrophoresis (CE).[11-15] Especially, a variety of chiral stationary phases (CSPs) for HPLC/UV have extensively been used for enantioseparation of flavonoids with an achiral mobile phase. Among the CSPs, cyclodextrin (CD) bonded phase have been one of important chiral selectors in HPLC.[16,17] CDs are cyclic oligosaccharides consisting of D-glucopyranose units linked by α-1,4 glycosidic bonds. They also have both relatively hydrophobic internal cavity and hydrophilic external surface. Their unique geometries are supposed to allow for enantioseparation by CDs.

Although liquid chromatography coupled with mass spectrometry (LC/MS) operated with negative ion electrospray ionization (ESI) has recently been used to resolve some flavanone glycosides on carboxymethylated β-CD column[18] or an isoflavone metabolite, equol on a Chiralcel column,[19] no study has been reported on enantioseparation of flavonoids using chiral β-CD column by LC-ESI-MS/MS assay which detect chiral flavonoid products through MS/MS fragmentation. In the present study, a chiral LC-ESI-MS/MS method was applied to resolve physiologically important chiral flavonoids.

Chemical structures of chiral flavonoids tested in this study are shown in Figure 1. Recently, interests in the potential therapeutic uses, plant physiologies, and health benefits of chiral flavanones have increased.[20-22] Therefore stereospecific assay methods for effective separation of chiral flavanones are necessarily needed. In this study, flavanone (2,3-dihydroflavone and naringenin), flavanone glycosides (naringin), and catechin were tested. Among the tested flavonoids, 2,3-dihydroflavone was stereospecifically separated with highest separation selectivity and resolution (Table 1). Firstly, an enantioseparation of 2,3-dihydroflavone was achieved by HPLC on a polysaccharide derivative cellulose trans-tris(4-phenylazaphenylcarbamate) column.[23] Using β-CD- or derivatized β-CD-bonded columns, the resolution of 2,3-dihydroflavone enatiomers by HPLC was also established.[7]

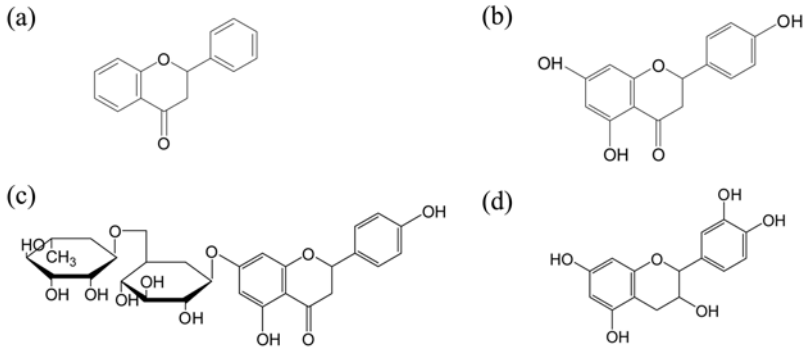

Figure 1. Chemical structures of chiral flavonoids, 2,3-dihydroflavone (a), naringenin (b), naringin (c), and catechin (d) tested in this study.

Table 1. Separation of chiral flavonoids on β-CD-bonded stationary phase by LC-ESI-MS/MS

	t_{m1}[a]	α[b]	R_s[c]
2,3-Dihydroflavone	7.72	1.20	2.13
Naringenin	6.66	1.01	0.12
Naringin	6.10	1.02	0.15
Catechin	11.24	1.05	0.38

[a]Migration times of the first isomers eluted from β-CD column. [b]Separation selectivity. [c]Resolution.

Figure 2(a) is a chromatogram showing resolution of 2,3-dihydroflavone enantiomers by LC-ESI-MS/MS (α = 1.20 and R_s = 2.13) under the mobile phase ratio of methanol and water of 80:20 (v/v). Furthermore, the relative abundances and the stoichiometries of the complex of free β-CD with 2,3-dihydroflavone formed in the mixture of water and methanol was determined in the gas phase. Major peak with most intense relative abundance was observed at *m/z* 1358, which corresponds to $[\beta\text{-CD+2,3-dihydroflavone-H}]^-$ ion (Figure 3(a)). Additionally, a minor peak was also observed at *m/z* 1246 corresponding to the 2:1 $([2\beta\text{-CD+2,3-dihydroflavone-2H}]^{2-})$ as the doubly charged ions. Regarding the structure of 2,3-dihydroflavone, an inclusion with the phenyl group or parts of the bicycle is possible.[7] This result indicates that 2,3-dihydroflavone enantiomers favorably form noncovalent interaction with β-CD and the noncovalent interaction can be a main force for the enantioseparation of 2,3-dihydroflavone by β-CD.

Naringin is the dominant flavonoid in grapefruits, and it gives a typical bitter taste. It has antioxidant, *anti*-carcinogenic and cholesterol lowering activity. On digestion, it releases its aglycone, naringenin. The naringenin and naringin enantiomers were separated with α = 1.01, R_s = 0.12 and α = 1.02, R_s = 0.15 by LC-ESI-MS/MS, respectively (Figure 2(b) and 2(c)). Additionally, major peaks corresponding to noncovalent complexes of β-CD/naringenin and β-CD/naringin were observed at *m/z* 1406 and *m/z* 1714, which indicate $[\beta\text{-CD+naringenin-H}]^-$ ion and $[\beta\text{-CD+naringin-H}]^-$ ion, respectively (Figure 3(b) and 3(c)).

In the case of catechin, a class of flavan-3-ol,[24-26] it has well been known that (+)-(2*R*;3*S*)-and (−)-(2*S*;3*R*)-catechin exhibit reverse effects on glycogen metabolism in isolated rat hepatocytes.[27] Also, (+)-(2*R*;3*S*)-catechin and (−)-(2*S*;3*R*)-catechin show antibacterial activity and allechemical activity, respectively, but their individual counterparts did not show any function.[25] For these reasons, chiral separation of catechin is needed. In this study, catechin resolved into its isomers by LC-ESI-MS/MS (α = 1.07, R_s = 0.98) under the mobile phase of methanol and water (80:20 (v/v)) (Table 1). The noncovalent complex of free β-CD with catechin formed in the mixture of water and methanol was observed as a major peak at *m/z* 1424 ($[\beta\text{-CD+catechin-H}]^-$ ion) in ESI-MS spectrum (Figure 3(d)). The higher complex ratios (e.g., 2:1, and 2:3) between β-CD and flavonoid (e.g., 2,3-dihydroflavone, catechin, and naringenin) in the negative ESI mass spectra of Figure 3 may be nonspecific and noncovalent adducts. The unusual binding mode between β-CD

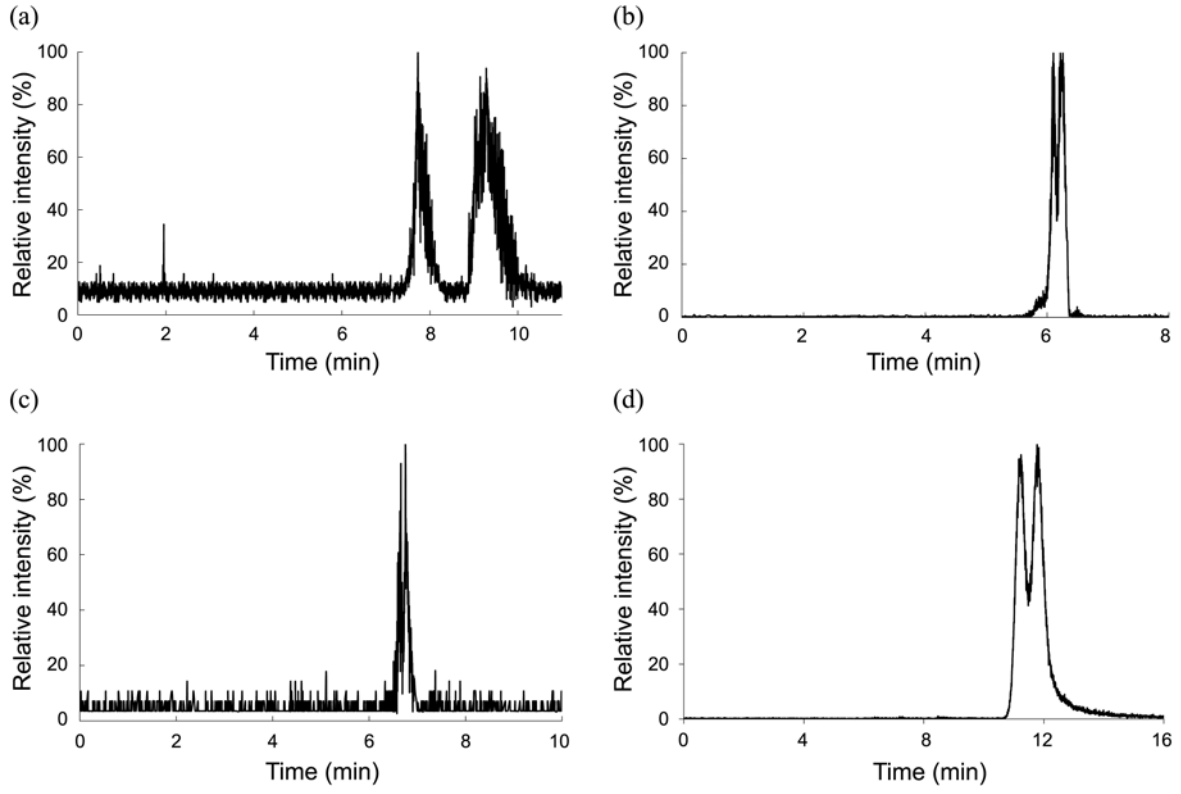

Figure 2. Chromatograms showing the enantioseparations of 2,3-dihydroflavone (a), naringenin (b), naringin (c), and catechin (d) by LC-ESI-MS/MS on β-CD-bonded chiral stationary phase.

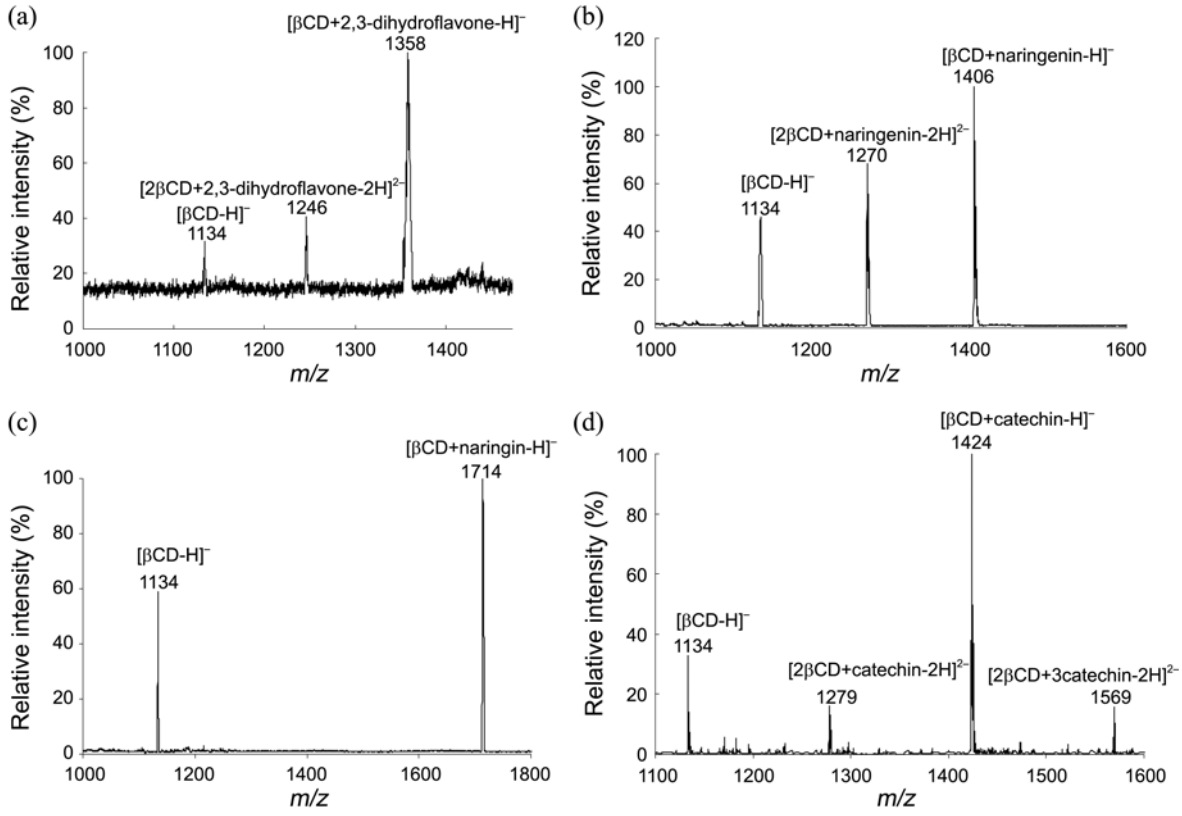

Figure 3. Negative ESI mass spectrum of the complex of β-CD with the racemates (2,3-dihydroflavone (a), naringenin (b), naringin (c), and catechin (d)) measured in a mixture of methanol with water.

and flavonoid in the gas phase may be different from that in solution phase. It has been reported that the ions corresponding to the higher complex are often detected as non-specific and noncovalent adducts during mass spectral analysis.[30-33]

Like above flavonoids, complex-forming ability of β-CD may be a main driving force for the chiral separation of catechin on β-CD-bonded stationary phase by LC-ESI-MS/MS. Actually, it has been reported that the benzene ring linked to the dihydropyran heterocycle of catechin is included into the secondary face of β-CD during the complexation, forming a noncovalent complex between β-CD and catechin, as evidenced by nuclear magnetic resonance spectroscopy (NMR) or molecular modeling study.[28,29]

In conclusion, the stereospecific resolutions of 2,3-dihydroflavone, naringenin, naringin and catechin were accomplished directly by LC-ESI-MS/MS on the β-CD-bonded stationary phase. Through this study, we showed that multiple reaction monitoring (MRM)-based LC-ESI-MS/MS methodology for the chiral separation of flavonoids on β-CD column would be an effective analytical tool. ESI/MS spectral analysis using Q1 scan mode suggested that stereoselective separation of chiral flavonoids tested in this study might occur through temporary formation of diastereoisomers on a β-CD-bonded chiral stationary phase with achiral mobile phase. Additionally, the present LC-ESI-MS/MS assay method might be of help to stereospecifically understand therapeutic effects, plant physiologies, and health benefits of chiral flavanones in some biological systems.

Experimental Section

Reagents. Racemates of 2,3-dihydroflavone, naringenin, naringin, and catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents including MeOH and water were of HPLC-grade and filtered through Advantec® membranes (pore size 0.45 μm, Toyo Roshi Kaisha, Ltd., Japan) before use.

Sample preparation. Each 1 mg of 2,3-dihydroflavone, naringenin, naringin, and catechin as racemates were dissolved in 80% MeOH solution (1 μL) and and each 1 mg of the flavonoids was injected into the chiral column on LC-ESI-MS/MS.

Preparation of the Complexes of β-CD with the Five Racemic Flavonoids. Each flavonoid was prepared at a concentration of 1 mM in methanol. Each 1 mL of the flavonoid solutions in a vial was mixed with 1 mL β-CD (1 mM) dissolved in water. The mixtures were stirred for 1h at room temperature before ESI mass analysis.

LC-ESI-MS/MS Experiment. All mass spectra were measured on a triple-quadruple mass spectrometer (API-4000™) equipped with a TurboIonSpray® probe (AB Sciex, CA, USA). The system included a series 1200 Agilent HPLC (Palo Alto, CA, USA) equipped with an auto-injector, antosampler, and quaternary pump. The following *m/z* multiple reaction monitoring (MRM) transitions were selected:

222.9 → 194.7 for 2,3-dihydroflavone, 271.0 → 118.9 for naringenin, 579.2 → 150.9 for naringin, and 289.1 → 123.0 for catechin. The instrument was operated in negative-ion mode with an ion-spray potential (IS) of −4500 V and source temperature of 650 °C For 2,3-dihydroflavone, DP, EP, and CXP were −85 V, −10 V, and −11 V, respectively. For naringenin, DP, EP, and CXP were −65 V, −10 V, and −9 V, respectively. For naringin, DP, EP, and CXP were −115 V, −10 V, and −7 V, respectively. For catechin, declustering potential (DP), entrance potential (EP), and collision cell exit potential (CXP) were −75 V, −10 V, and −5 V, respectively. The applied collision energies (CEs) were −26 eV for 2,3-dihydroflavone, −26 eV for naringenin, −58 eV for naringin, and −40 eV for catechin. Sample injection volume was 5 μL. Data were acquired and processed with Analyst™ 1.5 software. The chiral separations by LC-ESI-MS/MS were achieved on a Sumichiral OA-7000™ phase, β-CD column (4.6 mm × 250 mm, 5 μm, Phenomenex, U.S.A.).

The resolution (R_s) and separation selectivity (α) were calculated using the following equations, respectively:

$$R_s = 2(t_{m2} - t_{m1})/(W_1 + W_2)$$
$$\alpha = t_{m2} / t_{m1}$$

where t_{m1} and t_{m2} are the migration times of the first and the second isomers, W_1 and W_2 are the widths at the peak base of each isomer.

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