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Note

Chiral separation and discrimination of catechin by sinorhizobial octasaccharides in capillary electrophoresis and ^{13}C NMR spectroscopy

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ABSTRACT

Succinoglycan, a sinorhizobial exopolysaccharide produced by *Sinorhizobium meliloti*, is composed of an octasaccharide subunit. *S. meliloti* produces both high-molecular-weight and low-molecular-weight (M_r < 10,000) succinoglycans which consist of monomers, dimers, or trimers. Succinoglycan monomers were isolated and further purified in the monomer series (M1, M2, and M3) by the degree of succinylation. We used sinorhizobial octasaccharides (M1, M2, and M3) as chiral additives in capillary electrophoresis (CE) for chiral separation of catechin and also as chiral shift reagents with ^{13}C NMR spectroscopy for chiral discrimination of catechin. Chiral separation of catechin took place when sinorhizobial octasaccharides (M2 and M3) were added to the background electrolyte (BGE) in CE. NMR signal splittings were also observed in the interactions of sinorhizobial octasaccharides with the enantiomers of catechin. Both chiral separation and discrimination of catechin depend on the presence of succinate substituents of the linear monomeric octasaccharide in CE and NMR spectroscopy, suggesting that succinylation of sinorhizobial octasaccharide is decisive for the effective chiral separation and discrimination of catechin.

Succinoglycan, produced by *Sinorhizium meliloti* Rm1021, is a well-characterized acidic exopolysaccharide polymer of repeating octasaccharide subunits.[1] Succinoglycan is composed of octasaccharide subunits, which consist of one galactose and seven glucose residues joined by β-glycosidic linkages. The analyses with nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) have confirmed the structural details of the octasaccharide subunit.[2] *S. meliloti* produces both high-molecular-weight (HMW; M_r > 10,000) and low-molecular-weight (LMW; M_r < 10,000) succinoglycans. Wang et al. reported that the LMW fraction of succinoglycan is composed of monomers, dimers, and trimers of the succinoglycan octasaccharide with considerable heterogeneity of noncarbohydrate substitutions (acetyl, pyruvate, and succinate).[3] LMW succinoglycan is also involved in the symbiotic interaction between *Sinorhizobium* species and its specific symbiotic plant.[3–6] Throughout this interaction, LMW succinoglycan is suspected of being involved in complexation with various plant flavonoids.[7,8]

Catechins are catechol derivatives mainly found in green and black tea and in red wine, and they show various physiological effects such as anticarcinogenic, antiallergic, antiatherogenic, antibacterial, and antiviral activities.[9–14] Catechins are also chiral compounds that are prospective anticancer drug candidates (Fig. 1A). Due to the different physicochemical and biological characteristics of each enantiomer of a chiral compound, its chiral separation and discrimination are very important. As for example, it was reported that (+)- and (−)-catechin showed opposite effects on glycogen metabolism in isolated rat heptocytes.[15] (+)-Catechin was shown to be more bioavailable than (−)-catechin in one study.[16] Recently Bais et al. reported that (−)-catechin, but not (+)-isomer, had allelochemical activity and that (+)-catechin, but not (−)-isomer, showed the antibacterial activity.[17] Recently, enantiomeric separations of catechin and epicatechin by means of HPLC[18–22] or capillary electrophoresis (CE)[23–29] that used various cyclodextrins as chiral additives have been reported.

In the present study, succinoglycan monomers were isolated and further purified to monomer series (M1, M2, and M3) by the degree of succinylation (Fig. 1B). We used these sinorhizobial octasaccharides (M1, M2, and M3) as chiral additives in CE for the chiral separation of catechin. We also investigated the chiral discrimination of catechin by using monomer series as a chiral shift reagent for ^{13}C NMR spectroscopy.

The isolation and purification of sinorhizobial octasaccharides were carried out by ethanol precipitation methods and by chromatographic techniques using size-exclusion chromatography and anion-exchange chromatography. The structures of the sinorhizobial octasaccharides isolated from *S. meliloti* were confirmed through ^{1}H and ^{13}C NMR spectroscopy (Fig. 1B) as reported previously.[3] The molecular weight of sinorhizobial octasaccharides was also determined with triple quadrupole tandem mass spectrometry equipped with an electrospray-ionization (ESI) source. Depro-

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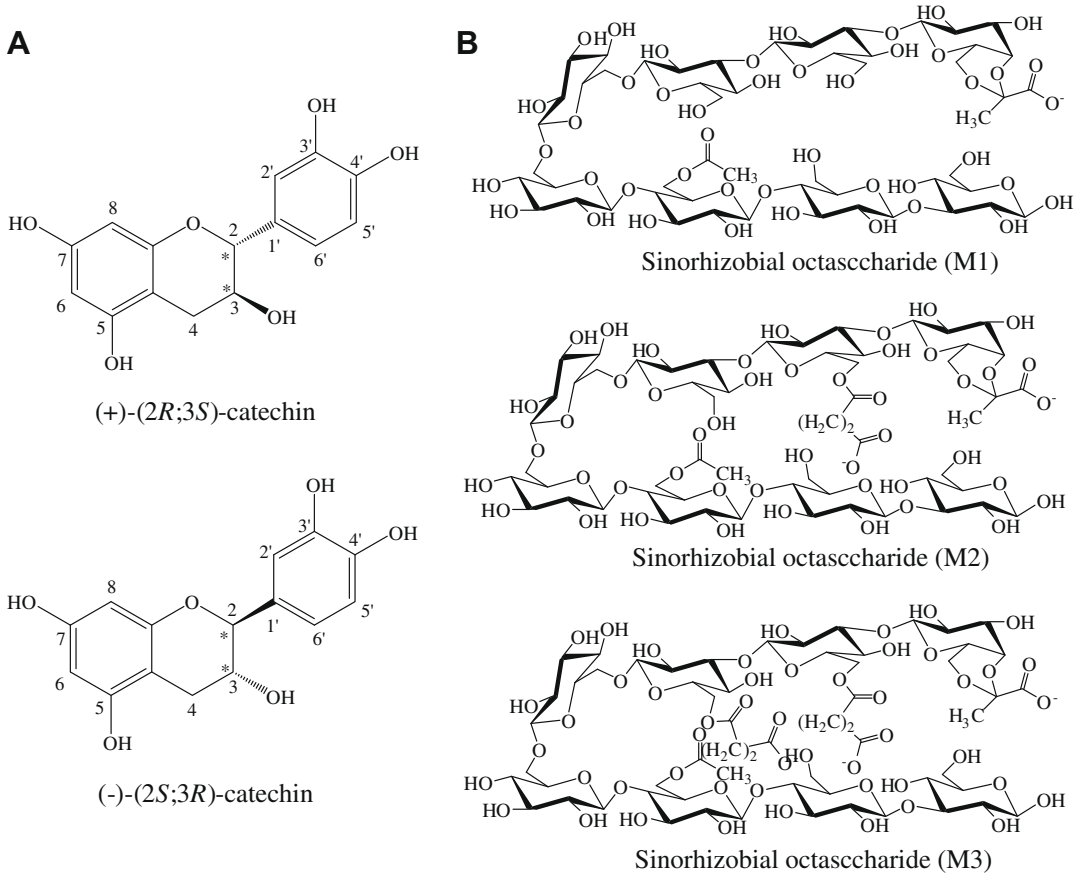

Figure 1. Chemical structures of (A) catechin and (B) succinoglycan monomers involved in this work.

tonated ions of the succinoglycan monomers in the negative ion mode were observed at *m/z* 1425, 1525, and 1625 corresponding to the molecular masses $[M1-H]^-$, $[M2-H]^-$, and $[M3-H]^-$ of the monomers M1, M2, and M3, respectively.

Various CE conditions such as concentration of chiral additives, pH, voltage, and temperature were investigated to accomplish the optimum chiral separation of catechin. The effect on addition of 50 mM sinorhizobial octasaccharides to the running buffer for the chiral separation of catechin was tested with various buffer solutions including 100 mM borate (pH 8.8, 9.5, and 10.5) and 50 mM phosphate (pH 7.5) buffer. The pH 8.8 value was chosen for effective chiral separation. At pH 7.5, no chiral separation of catechin was observed with sinorhizobial octasaccharides, which is probably due to the fewer negative charges on catechin at this pH.[30] No peak was observed within 60 min at pH 9.5 and 10.5. The low EOF due to the strong interactions between the sinorhizobial octasaccharides and catechin would prevent the analytes from reaching the detector.

The effect of voltage and temperature was investigated at pH 8.8. The maximal voltage output available with a 30 kV power supply was used for a short running time. However, 25 kV was determined to result in the optimal yields with the best selectivity and resolution. We also investigated optimal operation temperature in the range of 10–30 °C. With 50 mM sinorhizobial octasaccharide (M2) as the chiral additive, the enantioselectivity of catechin was obtained as 1.07 at 10 °C, 1.05 at 20 °C, and 1.02 at 30 °C. However, the migration time of catechin rapidly increased as the temperature decreased. An optimal temperature of 20 °C was selected in a compromise between lower migration times and higher resolutions since it provided reasonable run time (t_{m2} = 16.21) and enantioresolution (R_s = 1.78) (Table 1).

Various concentrations of sinorhizobial octasaccharides ranging from 10 to 100 mM were also investigated for the optimal enantioseparation of catechin in a fused-silica capillary with a BGE in 0.1 M borate buffer, pH 8.8 at 20 °C and 25 kV. No chiral separation of catechin was observed with a BGE containing 50 mM or 100 mM nonsuccinylated octasaccharide (M1). However, the enantiomers of catechin were effectively separated in the presence of M2 or M3 as the chiral additive in CE. However, enantiomers of catechin could be separated with the lower concentrations of M3 (40 mM; R_s = 3.84) as compared to M2 (60 mM; R_s = 2.15) (Table 1). These results show that both the presence and the number of succinate moieties on the linear octasaccharides play an important role in enantioseparation. The succinate moieties apparently support the characteristic conformation or facilitate hydrogen bonding necessary for chiral separations although the exact molecular mechanism remains to be determined. Figure 2 shows the best enantioseparation of catechin at 60 mM concentration of M2 (Fig. 2A) and 30 mM concentration of M3 (Fig 2B) with an optimal additive concentration with the BGE in CE.

^{13}C NMR experiments were conducted in order to obtain a better understanding of the mechanism by which enantiomers of catechin are discriminated in liquid phases that contain a sinorhizobial octasaccharide as the novel chiral shift reagent. The NMR signals observed in binary selector–solute solutions are time-averaged signals of both the complexed and uncomplexed analytes coupled with the monomer series (M1, M2, and M3) on shift nonequivalence, respectively. These chiral splitting phenomena were observed on the ^{13}C NMR spectra when sinorhizobial octasaccharides were mixed with the enantiomers of catechin (Fig. 3). Due to the different interactions between enantiomers of catechin and sinorhizobial octasaccharides (M1, M2, and M3), the reso-

Table 1
Influence of the type of sinorhizobial octasaccharides on the chiral separation of catechin[a]

Type of linear octasaccharides	Concentration of additive (mM)	t_{m1} (min)	t_{m2} (min)	α	R_s
M1	25	3.15	ns	ns	ns
	50	6.42	ns	ns	ns
	100	15.79	ns	ns	ns
M2	40	8.60	8.83	1.02	0.73
	50	15.41	16.21	1.05	1.78
	60	18.70	19.84	1.06	2.15
	80	14.29	15.07	1.05	1.90
M3	15	10.50	10.55	1.00	ns
	25	19.29	20.48	1.06	2.14
	30	22.04	23.85	1.08	3.09
	40	30.03	33.35	1.11	3.84

[a] Conditions: fused-silica capillary, 50.5 cm (effective length 42 cm) × 50 μm id; BGE, 100 mM borate buffer pH 8.8; UV detection at 280 nm; injection, 5 kPa pressure for 4 s; applied voltage, 25 kV, 20 °C; ns, no enantioseparation (R_s < 0.2).

nances of the carbon signals (δ 83.8, 68.5, and 29.4, respectively) in positions 2, 3, and 4 in the catechin enantiomers were split (Fig. 3) in D_2O containing 30% CD_3OD. The signal of the split carbon was also broadened in the presence of sinorhizobial octasaccharides, which suggest that the enantiomers are bound to these octasaccharides, and then induced to undergo the enantioseparation. A change in chemical shifts ($\Delta\delta$ 0.04 and 0.05) was observed in the NMR spectrum at positions 3 and 4 of the catechin enantiomers in the presence of M1, although no chiral separation was accomplished in CE. More significant chemical shift changes were observed in the presence of M2 ($\Delta\delta$ 0.08 and 0.07) or M3 ($\Delta\delta$ 0.08 and 0.08) rather than of M1 ($\Delta\delta$ 0.04 and 0.05). In addition, a unique chemical shift ($\Delta\delta$ 0.02 and 0.03) at position 2 of catechin was observed in the presence of M2 and M3. These results suggest that the succinate moiety could be significantly involved in the recognition or discrimination for the enantioseparation of catechin.

In summary, we used sinorhizobial octasaccharides (M1, M2, and M3) as chiral additives in CE for the chiral separation of catechin, and we also investigated the use of these oligosaccharides as chiral shift reagents for the chiral discrimination of catechin in ^{13}C NMR spectroscopy. The chiral separation of catechin took place when sinorhizobial octasaccharides (M2 and M3) were added to the BGE in CE. NMR signal splittings were also observed on the interactions of sinorhizobial octasaccharides with the enantiomers of catechin. The chiral separation and discrimination of catechin depend on the presence of succinate substituents on the linear monomeric octasaccharides in both CE and NMR spectroscopy, suggesting that the succinylation of sinorhizobial octasaccharide is decisive for the effective chiral separation and discrimination of catechin.

1. Experimental

1.1. Chemicals and reagents

All chemicals, including each enantiomer or racemate of catechin were purchased from Sigma–Aldrich Chemical Co. (St. Louis, Mo, USA). The structure of enantiomeric catechin is presented in Figure 1A. D_2O (99.9 atom% D) and CD_3OD (99.8 atom% D) were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI, USA) for use in NMR spectroscopy.

1.2. Preparation of sinorhizobial octasaccharides

For the preparation of sinorhizobial linear octasaccharides, *S. meliloti 1021* was grown in 500 mL of GMS medium at 30 °C for 5 days. Isolation, purification, and structural analyses of linear octasaccharides from *S. meliloti 1021* were carried out as described previously.[3] Cells were removed by centrifugation (13,000*g* for 10 min), and culture supernatants were concentrated fivefold by rotary evaporation. Next, high-molecular-weight (HMW) succinoglycan was precipitated from concentrated supernatants by adding 3 volumes of ice-cold EtOH. HMW succinoglycan was then removed from the concentrated supernatants by centrifugation (12,000*g* for 10 min). The supernatant was once more concentrated fivefold by rotary evaporation, and low-molecular-weight (LMW)

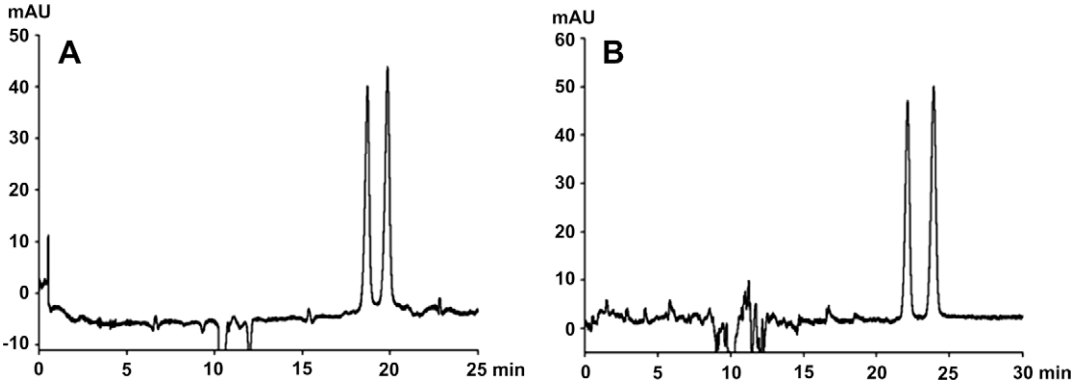

Figure 2. CE separation of catechin in the presence of (A) 60 mM succinoglycan monomer (M2), (B) 30 mM succinoglycan monomer (M3) used as a novel chiral additive in CE. Conditions: capillary, 50 cm (41.5 cm effective length) × 50 μm i.d.; 100 mM borate buffer pH 8.8; applied voltage, 25 kV; positive polarity at the inlet, 5 kPa pressure for 4 s; temperature, 20 °C; detection at 280 ± 10 nm.

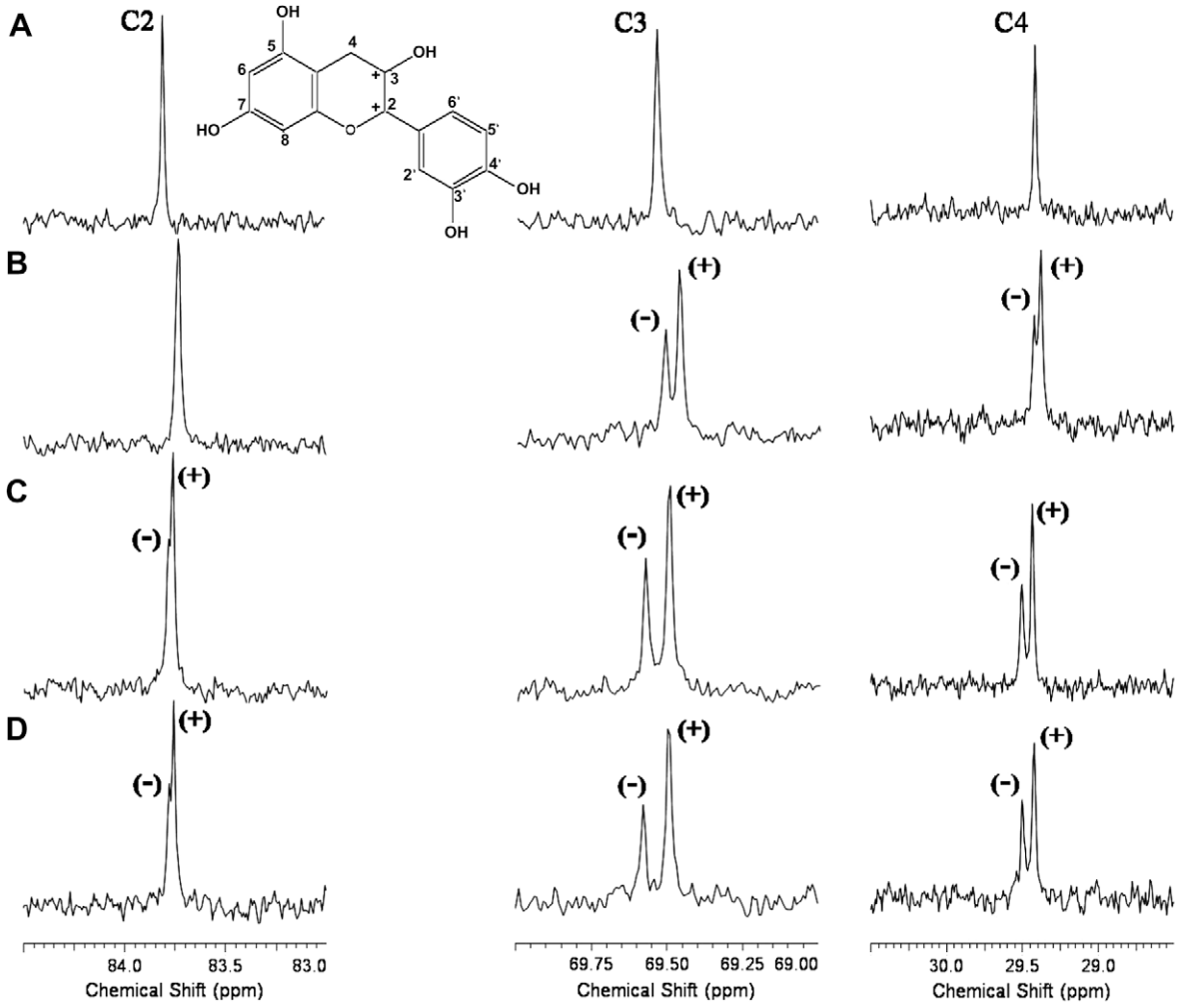

Figure 3. Partial ^{13}C NMR spectra of 20 mM nonracemic catechin (2:1 (+)–(−)) enantiomers in the (A) absence and (B) presence of 20 mM succinoglycan monomers M1, (C) M2, and (D) M3.

succinoglycan was collected by adding 7 volumes of ice-cold EtOH followed by centrifugation.

The low-molecular-weight (LMW) succinoglycan was then purified from concentrated supernatants using gel-permeation chromatography as described previously.[3] The purified linear octasaccharides were confirmed through NMR spectroscopy (Bruker 500 MHz) and triple quadrupole tandem mass spectrometry.

1.3. Capillary electrophoretic conditions

All capillary electrophoretic experiments were performed on an Agilent 3-D CE System (Wilmington, DE, USA) equipped with a diode array detector. Separations were carried out on uncoated 50 μm ID fused-silica capillary with a total length of 50.5 cm and an effective length of 42 cm to the detector window. The capillary was conditioned by flushing with 0.1 M NaOH for 20 min and then rinsed with water for 10 min, and finally equilibrated with an appropriate running buffer for 3 min. Between two runs, the capillary was rinsed with 0.1 M NaOH, water, and running buffer for 3 min each. The BGE consisted of an aqueous solution of 100 mM borate buffer (pH 8.8). The chiral additive buffer solutions were prepared by dissolving the linear octasaccharides in the BGE. The sample solutions were prepared in a running 9:1 buffer–MeOH mixture to a final concentration of 0.1 mg/mL, with introduction into the capillary using a pressure of 5 kPa for 4 s. A voltage of 25 kV was applied with the positive polarity at the inlet. The EOF was determined with MeOH as the neutral marker. Detection was done with on-column UV absorbance at 280 nm.

1.4. NMR spectroscopic analysis

Catechin 20 mM, sinorhizobial octasaccharides (M1, M2, and M3) 20 mM, and the nonracemic mixture (2:1 (+)/(−)) were prepared in H_2O containing 50% MeOH. Each mixture was stirred for 12 h under darkness, followed by degassing before NMR measurements. After reaction, each mixture was lyophilized and prepared in D_2O containing 30% CD_3OD for analysis by NMR spectroscopy.

NMR spectroscopic analysis was carried out on a Bruker AVANCE 500 spectrometer for sinorhizobial octasaccharides (M1, M2, and M3) and their complexes. The ^{13}C NMR spectra (125.7 MHz) were recorded in D_2O containing 30% CD_3OD. The instrument was equipped with a 5-mm probe, and all chemical shifts are referred to internal tetramethylsilane (TMS). Chemical shifts were calibrated with an accuracy of 0.05 ppm.

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References

1. Chouly, C.; Colquhoun, I. J.; Jodelet, A.; York, G.; Walker, G. C. *Int. J. Biol. Macromol.* **1995**, *17*, 357–363.
2. Reinhold, B. B.; Chan, S. Y.; Reuber, T. L.; Marra, A.; Walker, G. C.; Reinhold, V. N. *J. Bacteriol.* **1994**, *176*, 1997–2002.
3. Wang, L.; Wang, X. Y.; Pellock, B.; Walker, G. C. *J. Bacteriol.* **1999**, *181*, 6788–6796.
4. Gonztlez, J. E.; York, G. M.; Walker, G. C. *Gene* **1996**, *179*, 141–146.
5. Pellock, B. J.; Cheng, H.-P.; Walker, G. C. *J. Bacteriol.* **2000**, *182*, 4310–4318.
6. Staehelin, C.; Forsberg, L. S.; D'Haeze, W.; Gao, M. Y.; Carlson, R. W.; Xie, Z. P.; Pellock, B. J.; Jones, K. M.; Walker, G. C.; Streit, W. R.; Broughton, W. J. *J. Bacteriol.* **2006**, *188*, 6168–6178.
7. Hartwig, U. A.; Joseph, C. M.; Phillips, D. A. *Plant Physiol.* **1991**, *95*, 797–803.
8. Gonzalez, J. E.; Reuhs, B. L.; Walker, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8636–8641.
9. Yáñez, J. A.; Remsberg, C. M.; Miranda, N. D.; Andrews, P. K.; Vega-Villa, K. R.; Davies, N. M. *Biopharm. Drug Dispos.* **2008**, *29*, 63–82.
10. Katiyar, S. K.; Elmets, C. A. *Int. J. Oncol.* **2001**, *18*, 1307–1313.
11. Jung, Y. D.; Ellis, L. M. *Int. J. Exp. Pathol.* **2001**, *82*, 309–316.
12. Kazi, A.; Smith, D. M.; Daniel, K.; Zhong, S.; Gupta, P.; Bosley, M.; Dou, Q. P. *In Vivo* **2002**, *16*, 397–403.
13. Yang, C. S.; Maliakal, P.; Meng, X. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 25–54.
14. Lambert, J. D.; Yang, C. S. *Mutat. Res.* **2003**, *523*, 201–208.
15. Nyfeler, F.; Moser, U. K.; Walter, P. *Biochim. Biophys. Acta* **1983**, *763*, 50–57.
16. Donovan, J. L.; Crespy, V.; Oliveira, M.; Cooper, K. A.; Gibson, B. B.; Williamson, G. *Free Radical Res.* **2006**, *40*, 1029–1034.
17. Bais, H. P.; Walker, T. S.; Stermitz, F. R.; Hufbauer, R. A.; Vivanco, J. M. *Plant Physiol.* **2002**, *128*, 1173–1179.
18. Dalluge, J. J.; Nelson, B. C.; Thomas, J. B.; Sander, L. C. *J. Chromatogr., A* **1998**, *793*, 265–274.
19. Fernandez, P. L.; Martin, M. J.; Gonzalez, A. G.; Pablos, F. *Analyst* **2000**, *125*, 421–425.
20. Arts, I. C.; van de Putte, B.; Hollman, P. C. *J. Agric. Food Chem.* **2000**, *48*, 1746–1751.
21. Subagio, A.; Sari, P.; Morita, N. *Phytochem. Anal.* **2001**, *12*, 271–276.
22. Lin, Y. S.; Tsai, Y. J.; Tsay, J. S.; Lin, J. K. *J. Agric. Food Chem.* **2003**, *51*, 1864–1873.
23. Horie, H.; Kohata, K. *J. Chromatogr., A* **1998**, *802*, 219–223.
24. Worth, C. C. T.; Wießler, M.; Schmitz, O. J. *Electrophoresis* **2000**, *21*, 3634–3638.
25. Lee, S.; Jung, S. *Carbohydr. Res.* **2002**, *337*, 1785–1789.
26. Pomponio, R.; Gotti, R.; Luppi, B.; Cavrini, V. *Electrophoresis* **2003**, *24*, 1658–1667.
27. Shuji, K.; Atsushi, Y.; Akinobu, M.; Hiroko, Y. *Electrophoresis* **2004**, *25*, 2892–2898.
28. Park, H.; Jung, S. *Electrophoresis* **2005**, *26*, 3833–3838.
29. Kofink, M.; Papagiannopoulos, M.; Galensa, R. *Eur. Food Res. Technol.* **2007**, *225*, 569–577.
30. Muzolf, M.; Szymusiak, H.; Gliszczyńska-Swigło, A.; Rietjens, I. M.; Tyrakowska, B. *J. Agric. Food Chem.* **2008**, *56*, 816–823.