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Quantification of Nobiletin and Tangeretin in Citrus by Micellar Electrokinetic Capillary Chromatography

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A micellar electrokinetic capillary chromatographic (MEKC) method was developed for the two major polymethoxylated flavones, nobiletin and tangeretin, using a fused-silica open-tubular capillary in a 70% methanol extract from citrus albedo tissue. A MEKC separation buffer (pH 7.0), consisting of 32 mM sodium dodecyl sulphate (SDS), 20 mM sodium dihydrogen phosopate, 12 mM disodium tetraborate and 20% methanol, was selected in order to optimize separation conditions. The order of elution was nobiletin and tangeretin. Of the 46 species and cultivars in the *Citrus* genus, 17 were assayed for nobiletin and/or tangeretin in this study. The Acrumen and Pseudofortunella sections, classified by Tanaka, were found to contain the highest flavone concentrations.

Keywords: micellar electrokinetic capillary chromatography (MEKC), nobiletin, tangeretin, polymethoxylated flavone, capillary chromatography (CE)

Introduction

Numerous studies have demonstrated that increased consumption of fruits and vegetables reduces the risk of cancer (Steinmetz and Potter, 1991; Bolcke *et al.,* 1992). It has been suggested that certain phytochemical compounds contained in fruits and vegetables inhibit or reverse the process of cancer development (Steele *et al.,* 1994; Tanaka, 1994). Citrus fruits are considered to be rich sources of various bioactive phytochemical compounds that have anti-cancer and anti-inflammatory effects (Murakami *et al.,* 2000; Gould, 1997). Polymethoxylated flavones, such as nobiletin and tangeretin, which have several methoxyl groups in the parent molecule, are unique flavones found in citrus fruits (Kawaii *et al.,* 1999a and 1999b).

Ishiwa *et al.* (1999) demonstrated that nobiletin effectively down-regulates the production of pro-matrix metalloproteinase and PGE_2, and that it interferes with the proliferation of synovial fibroblasts. This suggests that it could be applied to maintaining articular cartilage and decrease pannus formation in rheumatoid arthritis and osteoarthritis. Nobiletin has been shown to have an inhibitory effect on phorbol ester-induced skin inflammation, oxidative stress, and tumor proliferation in mice (Murakami *et al.,* 2000), and decreasing condensation and sedimentation of red blood cells in vitro (Chen *et al.,* 1997). Tangeretin has been reported to efficiently induce apotosis in HL-60 cells (Hirano *et al.,* 1995). Furthermore, nobiletin and tangeretin exhibited differentiation-inducing activity toward mouse myeloid leukemia cells (M1), with cells exhibiting phagocytic activity (Sugiyama *et al.,* 1993). Given the above findings, the demand for polymethoxylated flavones has increased in recent years.

Previous reports have identified polymethoxylated flavones, such as nobiletin and tangeretin, in citrus tissues by high-performance liquid chromatography (HPLC) using a normal phase column (Bianchini and Gaydou, 1981) and a reverse phase column (Bianchini *et al.,* 1987; Nogata *et al.,* 1994). However, these techniques have primarily been applied to the analysis of polymethoxylated flavones in citrus oil.

Micellar electrokinetic capillary chromatography (MEKC) (Terabe *et al.,* 1984; Terabe *et al.,* 1985), a modified capillary electrophoretic (CE) technique, has been developed into a very powerful and efficient method for the separation of various charged and uncharged compounds (Nishi *et al.,* 1989; Otsuka and Terabe, S., 1990, Moodley *et al.,* 1995). In MEKC, a buffer solution containing a surfactant (e.g. sodium dodecyl sulphate: SDS) at its almost critical micelle concentration is used as the electrophoretic medium. This means that a psuedostationary phase is produced, enabling the separation of both charged and uncharged solutes in a single analysis according to differential distribution of the solutes between the aqueous and micellar phases. This capillary electrophoretic technique offered high separation ability and, in general, had a higher associated detection limit than HPLC. However, MEKC requires an injection in the order of nanoliters.

In the present study, we analyzed the major polymeth-

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oxylated flavones nobiletin and tangeretin by MEKC, and applied our findings to determine the nobiletin and tangeretin in citrus albedo tissues among 46 species.

Materials and Methods

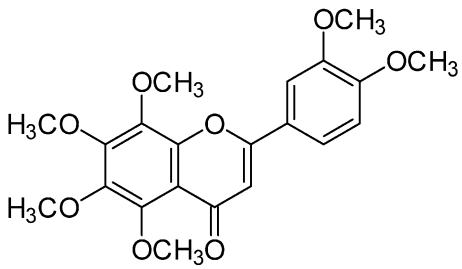
Materials Between 5 and 15 mature fruit of 46 citrus species were sampled from the trees at the Department of Citriculture, National Institute of Fruit Tree Science, Shizuoka, Japan, in December 2003. The washed fruit samples were peeled, and the albedo tissue (white parts) was removed from the peel and stored at −30℃. After lyophilization, albedo tissue samples were ground using an ultra centrifugal mill (Retsch mill, Brinkmann, Westbury, NY, USA) with a 0.5 mm filter, and stored at −80℃ until used (Nogata *et al.*, 2006). For the extraction solvent for nobiletin and tangeretin from citrus tissues, the mature fruits of Shiikuwasha (*Citrus depressa* Hayata), grown in the Agricultural Experiment Station of Okinawa Prefecture, Nago, Okinawa, Japan, on January in 2003, were used and albedo tissue samples were prepared as described.

Chemicals and standards of nobiletin and tangeretin Sodium dodecyl sulfate (SDS), sodium dihydrogen phosopate (NaH_2PO_4), and disodium tertraborate ($Na_2B_4O_7$) of specific analytical grade were purchased from Wako Pure Industries (Osaka, Japan). Distilled water was purified with Milli-Q system (Millipore Japan, Tokyo, Japan). All chemicals were of analytical-reagent grade and were used without further purification. The structures of nobiletin and tangeretin are shown Fig. 1.

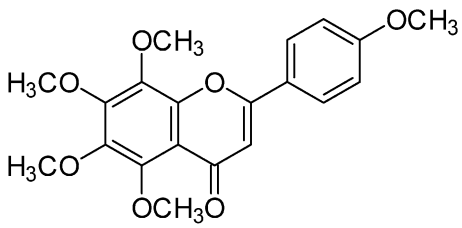
The standards for nobiletin and tangeretin were isolated from King juice (*Citrus nobilis*) and characterized by NMR spectrometry at the National Institute of Fruit Tree Science, Shizuoka, Japan, as described previously (Kawaii *et al.*, 1999a). All standards were dissolved in methanol (MeOH) and filtered though a 0.45-μm membrane filter (Advantech Toyo, Tokyo, Japan) prior to injection.

Apparatus for CE Capillary electrophoretic (CE) analysis was performed on a Quanta 4000E capillary electrophoresis system (Waters, Milford, MA, USA). A fused-silica open-tubular capillary (60 cm × 75 μm I.D.) was purchased from Waters (Milford, MA, USA). The electropherograms were analyzed on a NEC PC-9800 ns/a computer (Tokyo, Japan), which had 805 data stations software (Waters) recorded using a LaserShot A404 printer (Canon, Tokyo, Japan).

Extraction of nobiletin and tangeretin from albedo citrus tissues with MeOH-DMSO or 70% MeOH For the MeOH-DMSO (1 : 1) extraction, 200 mg samples of the lyophilized albedo powder were extracted in 5 mL of MeOH-DMSO (1 : 1) before being homogenized using a Model PT 10–30 Polytron homogenizer for 1 min (Kinematica, Littan/Luzern, Switzerland). The homogenate was then subjected to supersonic treatment for 30 min and incubated at ambient temperature for 10 h. After centrifugation at 4,000 g for 10 min, the supernatant was removed and the pellet was extracted once more in 3 mL of the same solution and centrifuged at 12,000 g for 15 min. The combined supernatant was then filtered through a 0.8-μm filter (Advantech Toyo, Tokyo, Japan) and adjusted to 10 mL in volumetric flask with distilled water filtered through a 0.45-μm membrane filter (Advantech Toyo) before the sample was subjected to MEKC.



Nobiletin



Tangeretin

Fig. 1. Structures of nobiletin and tangeretin.

For the 70% MeOH extraction, 250 mg samples of the lyophilized albedo powder were extracted in 5 mL of 70% MeOH, homogenized, and sonicated. After centrifugation at 4,000g for 10 min, the supernatant was removed, and the pellet was extracted once more in 3 mL of the same solution, and then centrifuged at 12,000 g for 15 min. The combined extract was then adjusted to 10 mL in a volumetric flask with 70% MeOH and filtered through a membrane filter (0.45-μm) prior to injection.

All treatments were performed in quadruplicate and all data are presented as average values ±SD. Comparisons were performed using Student's t test. A p value of less than 0.05 was regarded as indicating significance.

MEKC conditions MEKC with SDS was used to provide highly efficient separations and was conducted at ambient temperature and a constant voltage of 21 kV. A detector monitored the eluent at 214 nm. A fused-silica capillary (75 μm I.D.) with a length of 60 cm (54.5 cm from the injection end to the detector window) was used. Hydrostatic injections were used, with the positive end of

the tube inserted into a vessel containing a sample solution. For 5 seconds, the level of the sample vessel was raised by 9.6 cm in comparison to the collection of the electrolyte at the collection end. A standard curve was determined for each of compound.

All treatments were performed in triplicate and the results presented represent average values.

Results and Discussion

Determination of MEKC conditions

Electrophoretic medium Since nobiletin and tangeretin are uncharged compounds, we attempted to separate these compounds using MEKC and SDS. Figure 2 shows the influence of the MeOH concentration in the electrophoretic medium on the migration times of tangeretin and nobiletin at a concentration of 32 mM SDS. The separation of these peaks was apparent beyond 10% of MeOH. A MeOH concentration of 20% gave a perfect separation of the peaks, indicating the relative migration time was 1.215.

Figure 3 shows the influence of SDS concentration in the electrophoretic medium on separation of the nobiletin and tangeretin peaks. No peak was detected at SDS concentrations of less than of 20 mM. As can be seen in the range of 20–50 mM SDS concentration, the best separation was obtained at 32 mM SDS and 20% MeOH.

From these results, we selected the 32 mM SDS −12 mM sodium tetraborate-20 mM sodium phosphate-20% MeOH buffer solution (pH 7.0) as the electrophoretic medium. Figure 4 shows a typical electrophoretogram for nobiletin (migration time of 34.1 min) and tangeretin (migration time of 41.5 min).

Linearity In order to characterize the relationship between the nobiletin and tangeretin levels, and the peak area using the above MEKC condition, varying amounts of standard solution were injected into the MEKC. All the graphs exhibited good linearity and obeyed Beer's law for the concentration range of 0.05 to 1.2 mg/mL. The regression equation $y=a\ x+b$, where x is the amount of polymethoxylated flavone (mg/mL), y is the peak area, and the correlation coefficient, (r), of polymethoxylated flavone are as follows: for nobiletin: $y=(5.645\times10^5)x-(1.077\times10^4)$ ($r=0.998$) and for tangeretin: $y=(7.282\times10^5)x-(2.712\times10^4)$ ($r=0.997$), respectively.

Comparisons of extraction solvent for citrus tissues MeOH-DMSO (1 : 1 v/v) has commonly been used as an extraction solvent for flavonoids from citrus tissues (Nogata *et al.,* 1994; Kawaii *et al.,* 1999a and 1999b; Nogata *et al*; 2006). However, little information is available regarding the relative efficacy of extraction solvent for extracting polymethoxylated flavones from citrus tissue. In order to select an optimal extraction solvent for nobiletin and tangeretin, particularly given that these polymethoxylated flavones have weaker polarities when compared to normal flavonoids, we assayed nobiletin and tangeretin levels in the extracts prepared from Shiikuwasha albedo tissue with 70% MeOH, and compared these with those obtained for MeOH-DMSO (1 : 1) (Fig. 5).

Quantities of nobiletin and tangeretin extracted using 70% MeOH were 23.9 mg/g (dried sample) and 17.1 mg/g, respectively, and 16.7 mg/g and 11.9 mg/g for the MeOH-DMSO (1 : 1) extraction. Compared to an extraction using DMSO-MeOH (1 : 1), nobiletin and tangeretin in the 70% MeOH extract were high concentration, suggesting a significant improvement in extraction efficiency ($p<0.01$). We selected the 70% MeOH as an extraction solvent for nobiletin and tangeretin from citrus tissue in future studies.

Nobiletin and tangertin concentrations in citrus albedo tissue by MEKC The 46 species designated by Tanaka who classified the *Citrus* genus into two subgenera, 8 sections, and 16 subsections, are listed in Table 1 (Tanaka, 1969). The nobiletin and tangeretin concentrations (mg/g dried sample) extracted from the citrus albedo of these

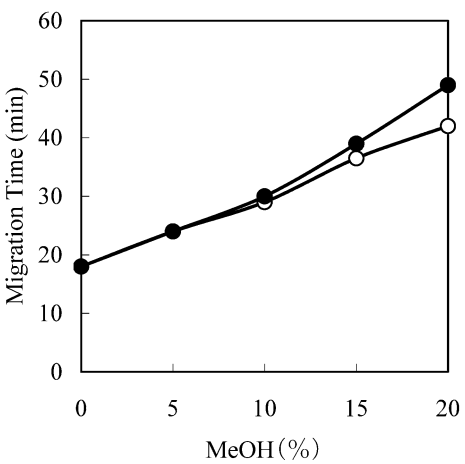


Fig. 2. Influence of MeOH concentration in the electrophoretic medium on the migration times of nobiletin (○) and tangeretin (●). Condition: 32 mM SDS.

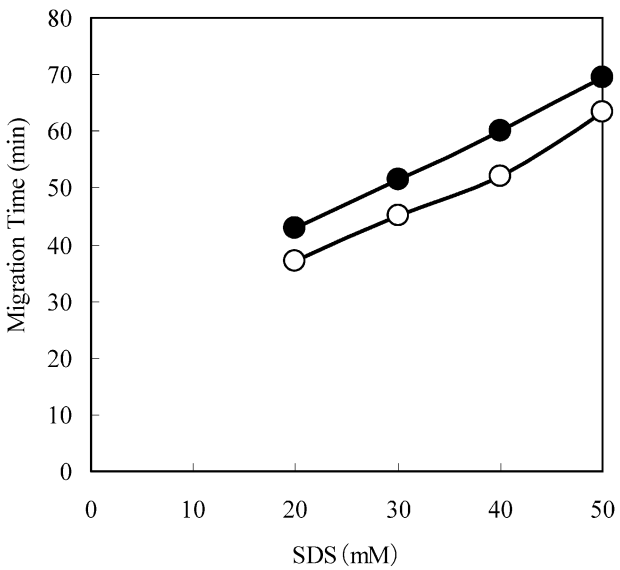


Fig. 3. Influence of SDS concentration in the electrophoretic medium on the migration of nobiletin (○) and tangeretin (●). No peak was detected at concentrations of less than 20 mM SDS.

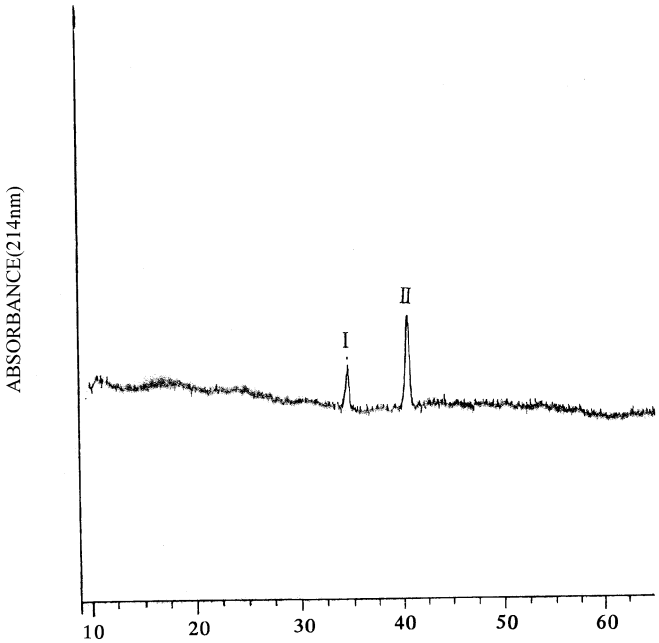


Fig. 4. Electrophoretogram of a mixture of nobiletin and tangeretin standards. Conditions: electrophoretic medium, pH 7.0 buffer solution (32 mM SDS-20 mM NaH_2PO_4–12 mM $Na_2B_4O_7$–20% MeOH); voltage, 21 kV; capillary, 54.5 cm (detection point), 60 cm (Total length)×75 μm I.D.; untreated fused-silica capillary. Peak: I =nobiletin (0.25 mg/mL); II=tangeretin (0.25 mg/mL).

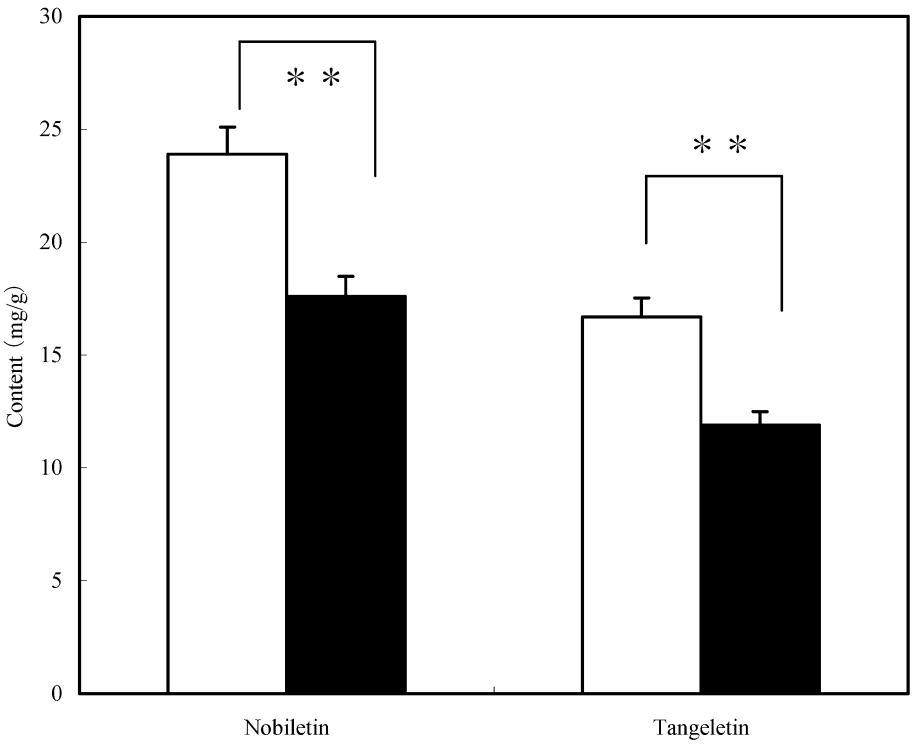


Fig. 5. Comparison of nobiletin and tangeretin concentrations extracted from the albedo powder of Shiikuwasha (*Citrus depressa*) with 70% MeOH (□) and MeOH-DMSO (1:1 v/v) (■). ** $p < 0.01$.

species by MEKC are also presented in Table 1.

As can be seen in Table 1, nobiletin and tangeretin were present at high concentrations in the *Acrumen* section, and at low concentrations in the *Aurantium* and *Psudo-fortunella* sections. In the *Acrumen* section, Shiikuwasha (*C. depressa*) contained the highest concentration of nobiletin among the 16 species assayed (47.5 mg/g dried sample). The distribution of tangeretin resembled that of nobiletin, with the highest concentrations found in Kishu (*C. kinokuni,* 27.9 mg/g), followed by Shiikuwasha

Table 1. Contents of nobiletin and tangeretin (mg/g dried sample) in albedo tissue of citrus fuit.

Classification of citrus based on Tanaka's classification				scientific name	conventional name	nobiletin	tangeretin
Aurantioideae	*Citrus*	*Archicitrus*	*Papeda*	*C. macroptera*	Kabuyao	n.d.	tr
			Limonellus	*C. aurantifolia*	Mexican lime	tr	tr
				C. latifolia	Tahiti lime	tr	n.d.
				C. bergamia	Bergamot	tr	n.d.
				C. montana	Biroro	2.5	0.9
			Citrioides	*C. medica*	Citron	n.d.	n.d.
				C. limon	Eureka lemon	n.d.	n.d.
				C. limetta	Sweet lemon	n.d.	tr
				C. lumia	Lumie	n.d.	n.d.
			Cephalocitrus	*C. grandis*	Hirado buntan	n.d.	n.d.
				C. grandis	Shaten yu	n.d.	n.d.
				C. paradisi	Marsh grapefruit	tr	n.d.
				C. glaberrima	Kinukawa	tr	tr
				C. hassaku	Hassaku	n.d.	n.d.
			Aurantium	*C. natsudaidai*	Natsudaidai	tr	tr
				C. sulcata	Sanbokan	tr	2.0
				C. aurantium	Shuto	tr	tr
				C. sinensis	Valencia	tr	tr
				C. sinensis	Morita navel	n.d.	n.d.
				C. iyo	Iyo	2.8	1.1
				C. tamurana	Hyuganatsu	tr	2.1
				C. shunkokan	Shunkokan	tr	1.6
		Metacitrus	*Osmocitrus*	*C. junos*	Yuzu	n.d.	n.d.
				C. sudachi	Sudachi	n.d.	n.d.
				C. sphaerocarpa	Kabosu	n.d.	n.d.
			Acrumen	*C. nobilis*	Kunenbo	2.1	2.0
				C. unshu	Unshu	1.8	1.9
				C. yatsushiro	Yatsushiro	3.0	3.8
				C. keraji	Keraji	25.2	24.4
				C. oto	Oto	2.9	2.6
				C. reticulata	Ponkan	19.6	15.6
				C. tangerina	Dancy tangerin	30.1	14.1
				C. succosa	Jimikan	6.4	4.9
				C. suhuiensis	Shikaikan	19.7	7.3
				C. clementina	Clementine	2.5	2.6
				C. tachibana	Tachibana	28.8	20.8
				C. erythrosa	Kobeni mikan	32.7	21.4
				C. kinokuni	Kishu	31.1	27.9
				C. sunki	Sunki	27.1	17.1
				C. depressa	Shiikuwasha	47.5	26.7
				C. leiocarpa	Koji	22.8	19.8
			Pseudofortunella	*C. madurensis*	Shikikitsu	4.9	2.2
	Fortunella			*F. margarita*	Nagami kunkan	n.d.	n.d.
				F.crassifolia	Ninpoukinkan	n.d.	n.d.
	Poncitrus			*P. trifoliata*	Karatati	n.d.	n.d.
	Severinia			*S. buxyfolia*	Tugekouji	n.d.	n.d.

tr: detected but too small to quantify, n.d.: not detected

(26.7 mg/g). These findings indicated that Shiikuwasha is a rich resource of nobiletin and tangeretin.

Conversely, nobiletin and tangeretin were lower than the detection limit or too small to be quantified in the *Cephacitrus* section, and were not detected in *Fortunella* and *Poncitrus* in this study.

In conclusion, we developed the first method for assaying the two major polymethoxylated flavones, nobiletin and tangeretin in citrus by MEKC. The present data confirm the applicability of MEKC to the detection and quantification of nobiletin and tangeretin in citrus albedo tissues. Nobiletin and tangeretin were found in samples from the Acrumen and Pseudofortunella sections as classified by Tanaka. The rinds of Shiikuwasha, Dancy tangerine (*C. tangerina*) and Keragi (*C. keraji*) were particularly rich sources of both nobiletin and tangeretin.

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