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Analytical Methods

Simultaneous determination of polymethoxyflavones in *Citrus* species, Kiyomi tangor and Satsuma mandarin, by high performance liquid chromatography

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ABSTRACT

The content of polymethoxyflavones (PMFs) in Kiyomi tangor (*Citrus unshiu* Marcov. forma *miyagawa-wase* × *C. sinensis*) and Satsuma mandarin (*C. unshiu* Marcov. forma *miyagawa-wase*) was determined by HPLC/UV. The major PMFs of Kiyomi tangor were determined as 3,6,7,4′-tetramethoxyflavone in the peels (10.1 mg/g) and leaves (9.2 mg/g), and 3-hydroxy-5,6,7,4′-tetramethoxyflavone in the stems (1.5 mg/g). The major PMFs of Satsuma mandarin were determined as 5,6,7,8,4′-pentamethoxyflavone in the peels (2.2 mg/g) and leaves (1.6 mg/g), and 5,6,7,3′,4′-pentamethoxyflavone in the stems (1.4 mg/g). Large amounts of *Citrus* by-products can ultimately provide a cheap and convenient source of PMFs.

1. Introduction

The genus *Citrus* is the most cultivated fruit in the subtropical latitudes of the northern hemisphere (Cooper & Chapot, 1977). Fruits of the *Citrus* species are universally important in over 100 countries, covering all six continents, because of its industrial value, especially in food and cosmetics (Choi & Min, 2004). Recently, some of the most grown *Citrus* species include the hybrid varieties, such as Kiyomi tangor (*C. unshiu* Marcov. forma *miyagawa-wase* × *C. sinensis* Osbeck) and Hallabong ([*C. unshiu* × *C. sinensis* Osbeck] × *C. reticulata* Blanco). Among them, Kiyomi tangor, a cross between Satsuma mandarin (*C. unshiu* Marcov. forma *miyagawa-wase*) and Trovita orange (*C. sinensis*), was first bred at the Shizuoka Branch of the Fruit Tree Research Station, Japan, in 1949. The flavour is similar to that of mandarin, while the aroma is similar to that of the orange (Nishiura et al., 1983). *Citrus* fruits are used for juice manufacturing, with peels produced as by-products. In some regions of the world, the peels of *Citrus* species are used in traditional herbal medicine to treat chronic diseases, such as coughing, upset stomach, and skin inflammation (Li et al., 2009).

Phytochemical investigations of *Citrus* species have shown that the peels contain flavonoids (Horie, Tsukayama, Yamada, Miura, & Nakayama, 1986), limonoids (Hasegawa, Raymond, & Zareb, 1986; Raymond, Miyake, Ozaki, & Hasegawa, 1991), coumarins (Tatum & Berry, 1979), and acridone alkaloids (Wu, Kuoh, & Furukawa, 1983). Among these compounds, polymethoxyflavones (PMFs) are major constituents in *Citrus* peels in addition to terpenoids and other volatile constituents (Kim, Hyun, & Ko, 1999; Min Tu, Thanh, Une, Ukeda, & Sawamura, 2002; Wada et al., 2007; Wang, Wang, Huang, Tu, & Ni, 2007). The PMFs in the category of flavonoid from the plant have shown to have significant biological activity, including antioxidant and antitumor properties (Jeong, Park, & Chung, 1997; Su, Shyu, & Chien, 2008; Yoshiki et al., 2008). The PMFs played a role in inhibiting the MEK and sequential ERK activities, which resulted in the suppression of the pro MMP-9 production in a cancer cell line (Miyata et al., 2007) and have higher antioxidant capacity (Kanaze et al., 2009; Xu et al., 2008). In spite of these previous studies, few studies have been conducted in phytochemical constituents from hybrid and parent *Citrus* species.

In this study, we evaluated the quantification of the PMFs in three parts of the hybrid species, Kiyomi tangor, and the parent species, Satsuma mandarin, using HPLC/UV.

2. Materials and methods

2.1. Plant materials

The peels, leaves, and stems of Kiyomi tangor (*C. unshiu* Marcov. forma *miyagawa-wase* × *C. sinensis* Osbeck, LEE 2009-04, 2009-05, and 2009-06, respectively) and Satsuma mandarin (*C. unshiu* Marcov. forma *miyagawa-wase*, LEE 2009-07, 2009-08, and 2009-09, respectively) were collected in Jeju, Korea. Six samples were sliced and dried in an oven at 45 °C until use. Voucher

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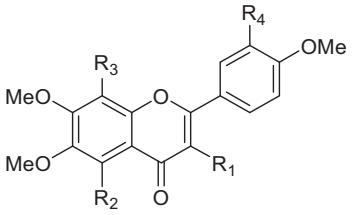

1 R_1 = OMe, R_2 = OMe, R_3 = OMe, R_4 = OMe

2 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = H

3 R_1 = H, R_2 = H, R_3 = OMe, R_4 = OMe

4 R_1 = OMe, R_2 = H, R_3 = H, R_4 = H

5 R_1 =H, R_2 = OMe, R_3 = H, R_4 = OMe

6 R_1 = OH, R_2 = OMe, R_3 = H, R_4 = H

7 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = OMe

Fig. 1. Chemical structures of compounds **1–7**.

specimens were deposited at the Herbarium of Department of Integrative Plant Science, Chung-Ang University, Korea.

2.2. Chemicals and instruments

Thin layer chromatography (TLC) was performed with precoated silica gel 60 F_{254} (Art. 5715, Merck Co., Germany) plates for the analysis. The stationary phase of repeated column chromatography was silica gel (No. 7734; Merck Co., Germany). High performance liquid chromatography (HPLC) chromatograms were recorded with a GILSON 305 system pump, using a GILSON 188 system UV/Vis detector (Villiers le Bel, France). For HPLC, first grade solvents such as water and ACN (J.T. Baker, US) were used as an elution solution, while all other reagents were analytical grade.

2.3. Preparation of standard compounds

In a previous work (Han, Kim, Lee, Mok, & Lee, 2010), seven PMFs, 5,6,7,3′,4′-pentamethoxyflavone (**1**), 6,7,8,3′,4′-pentamethoxyflavone (**2**), 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**3**), 5,6,7,8,3′,4′-hexamethoxyflavone (**4**), 3,6,7,4′-tetramethoxyflavone (**5**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**6**), and 5,6,7,8,4′-pentamethoxyflavone (**7**), were isolated and identified from the sliced and dried

Table 1
Linearity of standard curves for compounds **1–7**.

Compounds	t_R	Linear range (μg/mL)	Calibration equation[a]	Correlation factor, r^{2b}
1	26.9	0.048 – 3.125	$Y = 416623.2075X + 74.4273$	0.9995
2	29.7	0.024 – 1.562	$Y = 998450.0152X - 41.3359$	0.9993
3	30.0	0.048 – 3.125	$Y = 352925.4705X + 82.3960$	0.9991
4	32.0	0.012 – 3.125	$Y = 6066733.0582X - 48.4282$	0.9999
5	32.7	0.012 – 0.781	$Y = 661209.0053X + 12.3448$	0.9995
6	33.6	0.012 – 3.125	$Y = 3953208.1690X - 32.3843$	0.9997
7	35.7	0.012 – 3.125	$Y = 513832.4675X + 95.6221$	0.9994

[a] Y = peak area, X = concentration of standards (mg/mL).
[b] r^2 = correlation coefficient for five data points in the calibration curves (n = 12).

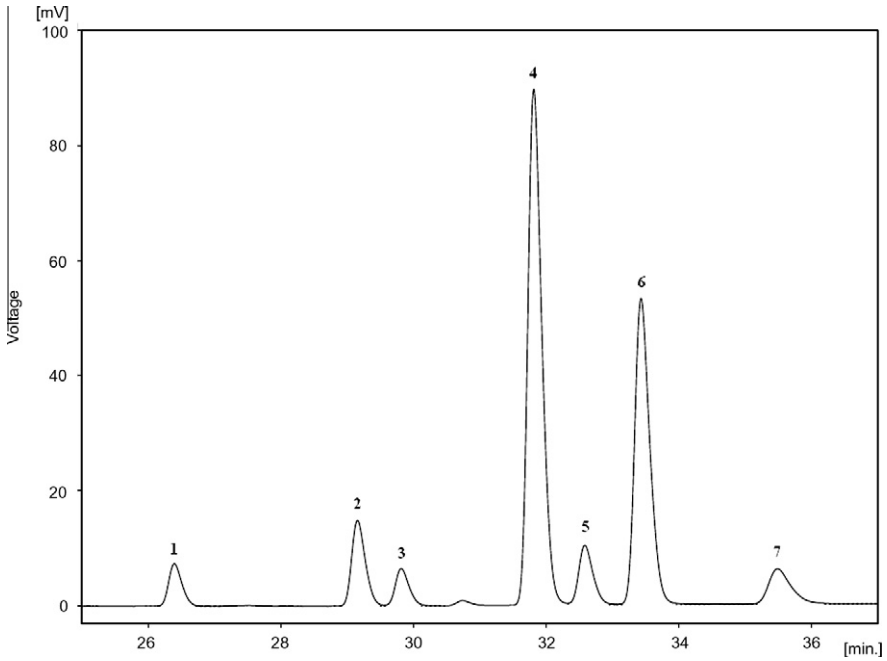

Fig. 2. HPLC chromatogram of PMF standard mixtures.