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Evaluation of bioactive flavonoids and antioxidant activity in Pericarpium Citri Reticulatae (*Citrus reticulata* 'Chachi') during storage

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Tangeretin (PubChem CID: 68077)
5-*O*-Desmethyl nobiletin (PubChem CID: 358832)
Catechin (PubChem CID: 73160)
1,1-Diphenyl-2-(2,4,6-trinitrophenyl)hydrazine (DPPH) (PubChem CID: 74358)
2,4,6-Tripyridyl-s-triazine (TPTZ) (PubChem CID: 77258)
2,2′-Azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS) (PubChem CID: 9570474)
6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (PubChem CID: 40634)

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Flavonoids
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ABSTRACT

A simple and accurate method using high performance liquid chromatography (HPLC) with dual wavelength detection was developed to simultaneously determine the contents of one flavanone glycoside (hesperidin) and five polymethoxylated flavones (PMFs: sinensetin, 4′,5,7,8-tetramethoxyflavone, nobiletin, tangeretin and 5-*O*-desmethyl nobiletin) in Pericarpium Citri Reticulatae (*Citrus reticulata* 'Chachi') ('Chachi' PCR). By modifying the mobile phase compositions and detection wavelengths, an optimal HPLC condition was obtained, under which the calibration curves of all six compounds exhibited good linearity ($R^2 > 0.99$). For all the tested compounds, the relative standard deviation (RSD) was less than 4%, and the accuracy ranged from 97.58 to 103.2%. The developed method was successfully applied to monitor the changes in the contents of six flavonoids in 'Chachi' PCR during storage at 25 °C, over a three year period. Color parameters and antioxidant capacity were also determined to evaluate the sample quality. The contents of hesperidin decreased while all the polymethoxylated flavones and antioxidant activities increased throughout the storage period, demonstrating that polymethoxylated flavones could be used as indices for the quality change of Chachi' PCR during storage. The results from this study suggest that the longer storage periods increased the quality of PCR.

1. Introduction

Pericarpium Citri Reticulatae (PCR, Chenpi in Chinese), the dried and matured tangerine pericarp, is one of the most famous Chinese herbal medicines officially listed in the Chinese Pharmacopoeia. It has been widely used for treating indigestion and inflammatory respiratory diseases such as bronchitis and asthma (Wang, Xu, Bin, Ling, & Chen, 2006). PCR is also often used as a health-care food in the folk tradition of southern China. It is found to be effective in resolving cough and phlegm, stimulating appetite, and enhancing immune system functions (Xu et al., 2014). PCR is usually added to foods as a condiment or sometimes used to regulate the taste of Chinese medicines because of its smell, flavor, and curative effects (Xia, Kotani, Hakamata, & Kusu, 2006). Among the main Chenpi cultivars, the dried ripe pericarp of *Citrus reticulata* 'Chachi' (Guang Chenpi in Chinese), grown in the Xinhui District of Guangdong Province, China, is regarded as the source of genuine PCR due to its excellent quality (Sun et al., 2010).

Phytochemical and pharmacological studies demonstrated that the major components of PCR are dietary flavonoids, which are generally categorized into two groups: flavanone glycosides (primarily hesperidin) and polymethoxylated flavones (PMFs, primarily nobiletin and tangeretin) (Ho & Kuo, 2014). Hesperidin is used

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as a chemical reference for quality control of PCR in the Chinese Pharmacopoeia because of its extremely high concentration (over 3%) and its anticancer (Devi et al., 2015), antioxidant (Pari, Karthikeyan, Karthika, & Rathinam, 2015), anticonvulsant (Dimpfel, 2006), and anti-inflammatory properties (Chang et al., 2015). PMFs are generally present as minor components in the aqueous extracts of *Citrus* plants, although they are more active than flavonoid glycosides (Xu et al., 2014). They are found to possess antioxidant (Xi, Fang, Zhao, Jiao, & Zhou, 2014), anticancer (Kim, Moon, Mosaddik, & Cho, 2010; Sergeev, Li, Colby, Ho, & Dushenkov, 2006), antiviral(Xu et al., 2014), antiangiogenic (Saito, Abe, & Nogata, 2015), antimicrobial (Wu et al., 2014) and anti-inflammatory (Green, Wheatley, McGrowder, Dilworth, & Asemota, 2013) activities.

To evaluate certain assertions in the Chinese Pharmacopoeia and folk traditions of southern China, PCR was characterized relative to its storage time in the present study. The folk proverb "PCR, the older, the better" suggests that storing PCR for longer periods improves its quality, which may be due to changes in the active ingredients during storage (Yi, Xie, Liang, & Lu, 2005). Quantifying the bioactive components of PCR during storage could confirm this claim. Several studies have quantified flavonoids in *Citrus* herbs using thin-layer chromatography (TLC) (Wang & Luo, 1989), HPLC-UV (Camarda, Di Stefano, Del Bosco, & Schillaci, 2007), HPLC-DAD (Zheng et al., 2009), HPLC-ECD (Careri, Elviri, Mangia, & Musci, 2000), HPLC-MS (Liu et al., 2013), and CE-ECD (Peng, Liu, & Ye, 2006), primarily focusing on the determination of flavonoids in fruits, juices or PCR of different *Citrus* species. However, little is known about changes in the bioactive flavonoids of PCR during storage. Thus, the objectives of this study were to develop a simple, accurate, and reliable HPLC method for the simultaneous determination of six bioactive flavonoids (Fig. 1), including hesperidin (**C1**), sinensetin (**C2**), 4′,5,7,8-tetramethoxyflavone (**C3**), nobiletin (**C4**), tangeretin (**C5**) and 5-*O*-desmethyl nobiletin (**C6**); and monitor the changes in the contents of these compounds in 'Chachi' PCR (Guangchenpi) samples during three years of storage. Color parameters and the antioxidant capacity were also determined to evaluate the sample quality. The results provide detailed information for identifying the storage stage of 'Chachi' PCR.

2. Materials and methods

2.1. Chemicals and materials

2.1.1. Chemicals

HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was used after filtration through a 0.45 μm membrane filter. 1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazine (DPPH) was purchased from Wako Co., Ltd (Kyoto, Japan). 2,4,6-Tripyridyl-s-triazine (TPTZ) was purchased from Tokyo Kasei Kogyo Co., Ltd. 2,2′-Azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Amresco Co., Ltd (Tokyo, Japan). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was obtained from Sigma (New York, America). Reference standards of flavonoids **C1-6** (hesperidin, sinensetin, 4′,5,7,8-tetramethoxyflavone, nobiletin, tangeretin and 5-*O*-desmethyl nobiletin) were isolated and purified from 'Chachi' PCR by conventional column chromatography, and their structures were identified by EI-MS, ^{1}H NMR, and ^{13}C NMR in comparison with the data from the literature. Their purities were determined to be 95% based on HPLC analysis using a peak area normalization method.

2.1.2. Preparation of plant material

Approximately 50 kg of fresh *C. reticulata* 'Chachi' fruit was collected from the Xinhui District, Guangdong Province, China in 2012. The citrus peels were removed and dried to a constant weight in a heat pump dryer (GHRH-20, Guangdong Agri-machinery Research Institute, China) at 45 °C.

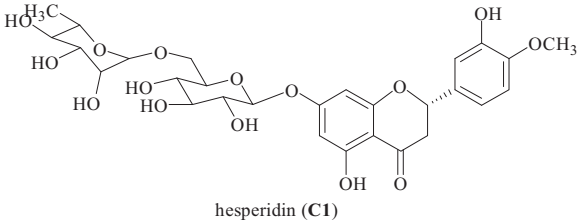
2.1.3. Sample packaging and storage conditions

Each 100.0 g samples of 'Chachi' PCR were packaged in aluminum foil laminate pot (Guangdong Weifu Packaging Material Co. Ltd., Guangdong, China) and stored at room temperature (25 ± 3 °C) and at a relative humidity of 60 ± 5% for use in future experiments. The experiments were replicated three times.

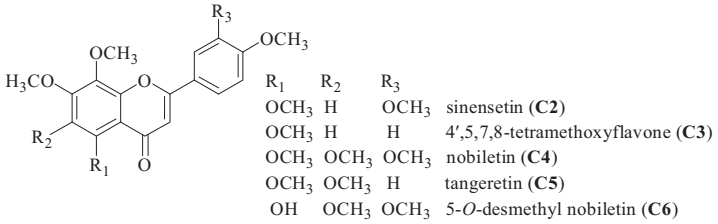
2.2. HPLC method

2.2.1. HPLC conditions

The flavonoids were detected at 280 nm and 330 nm by the UV detector using an Agilent 1260 Series HPLC system (Agilent Technologies Inc., USA). The compounds were separated on an Agilent ZORBAX SB C_{18} column (4.6 mm × 250 mm). The solvent system consisted of acetonitrile and water with 0.4% formic acid with the following gradient elution program: 0–15 min, 25–50% acetonitrile; 15–35 min, 50–60% acetonitrile; and 35–40 min, 60–85% acetonitrile. The mobile phase conditions were modified from a previously reported method (Zheng et al., 2009). The flow rate was 1.0 mL/min, and the injection volume of the sample was 10 μL. The effluent was monitored by UV detection at 280 nm for compound **C1** and 330 nm for compounds, **C2-6**.



hesperidin (**C1**)



R_1	R_2	R_3	
OCH_3	H	OCH_3	sinensetin (**C2**)
OCH_3	H	H	4′,5,7,8-tetramethoxyflavone (**C3**)
OCH_3	OCH_3	OCH_3	nobiletin (**C4**)
OCH_3	OCH_3	H	tangeretin (**C5**)
OH	OCH_3	OCH_3	5-*O*-desmethyl nobiletin (**C6**)

Fig. 1. Chemical structures of the bioactive flavonoids (**C1-6**).