

**DETERMINATION OF FLAVONOIDS IN CITRUS FIBER AND THE IMPACT OF PROCESSING METHOD ON TOTAL PHENOLIC CONTENT, TOTAL FLAVONOID CONTENT, TOTAL CAROTENOID CONTENT AND OXYGEN RADICAL ABSORBANCE CAPACITY**

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**ABSTRACT**

The objective of this study is to evaluate the presence of flavonoids in citrus fiber using reverse-phase high-pressure liquid chromatography (RP-HPLC). In this study, we also investigate the effect of regular and hot-washing preparations on total polyphenol content (TPC), total flavonoid content (TFC), oxygen radical absorbance capacity (ORAC) and total carotenoid content (TCC) of citrus fiber. The HPLC method used provided fast results and good separation for polymethoxyflavones and flavonols. Quercetin and kaempferol (flavonols), and sinensetin, nobiletin, heptamethoxyflavone, and tangeretin (polymethoxyflavones) were identified. Nobiletin had the highest concentration with 3.33 mg/g dry weight followed by sinensetin (1.96 mg/g dry weight) and heptamethoxyflavone (1.24 mg/g dry weight), respectively. The TFC and TCC of citrus fiber were unaffected ( $P > 0.05$ ) by the citrus fiber production procedure; however, the TPC and the ORAC values of citrus fiber prepared using hot-washing were significantly lower ( $P < 0.05$ ).

**Keywords:** Citrus, fiber, flavonoid, total polyphenol content, ORAC value, HPLC

**TURUNÇGİL LİFİNDE FLAVONOİDLERİN BELİRLENMESİ VE ÜRETİM METODUNUN TOPLAM FENOLİK MİKTARI, TOPLAM FLAVONOİD MİKTARI, TOPLAM KAROTENOİT MİKTARI VE OKSİJEN RADİKAL SOĞURMA KAPASİTESİNE ETKİSİ**

**ÖZ**

Bu çalışmanın amacı turunçgil kabuğundan elde edilen liflerin ters fazlı yüksek basınçlı sıvı kromatografisi yöntemi ile flavonoidlerinin belirlenmesidir. Ayrıca, bu çalışmada turunçgil kabuklarından lif hazırlanırken kullanılan normal veya sıcak yıkama işlemlerinin toplam fenol miktarı, toplam flavonoid miktarı, toplam karotenoid miktarı ve oksijen radikal soğurma kapasitesi değerine etkisi araştırılmaktadır. Kullanılan HPLC metodu çoklu metoksiflavon ve flavanollerin ayrılmasını sağlayan ve hızlı sonuç veren bir yöntemdir. Kuersetin, kemferol, sinensetin, nobiletin, heptamethoksiflavon ve tangeretin belirlenmiştir. Nobiletin 3.33 mg/g kuru ağırlık ile en yüksek miktarda bulunan flavonoiddir. Sinensetin 1.96 mg/g kuru ağırlık ile, heptamethoksiflavon ise 1.24 mg/g kuru ağırlık ile takip etmektedir. Sıcak yıkama veya normal yıkamanın toplam flavonoid içeriği ve toplam karotenoid içeriğine etkisi görülmemiştir ( $P > 0.05$ ). Ancak, toplam fenolik madde ve oksijen radikal soğurma kapasitesi değeri sıcak yıkama yapılan liflerde daha düşük bulunmuştur ( $P < 0.05$ ).

**Anahtar kelimeler:** Turunçgil, lif, flavonoid, toplam fenol miktarı, oksijen radikal soğurma kapasitesi değeri, HPLC

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## INTRODUCTION

Citrus fruit production takes place mostly in tropical and sub-tropical areas in the world. Main citrus fruits are oranges, grapefruit, lemon, tangelos, tangerines and mandarins. According to a U. S. Department of Agriculture (2013) report approximately 35.5% of the citrus fruits are consumed as fresh fruit while the rest has been processed into juice. Using the majority of the citrus fruits for juice production creates a large amount of waste. The waste products from the peel and seeds are prone to microbial spoilage, making this waste material a candidate for use mostly as animal feed and fertilizer (Fernandez-Lopez et al., 2004). However, it has been reported by Saura-Calixto (1998) and Larrauri (1999) that fruit by-products, such as peels, are rich in dietary fiber. Also, due to the presence of flavonoids and carotenoids, they have better nutritional quality than fiber from cereals, as well as a balanced composition of soluble and insoluble fiber and low energy values. Also, Gorinstein et al. (2001) and Fernandez-Lopez et al. (2004) stated that citrus by-products, such as peel (albedo and flavedo), are not only rich in fiber, but due to the presence of phenolic compounds and antioxidants, such as ascorbic acid, they can be used in food products to provide further functional benefits. Lario et al. (2004) stated that fruit fibers, such as citrus, provide good quality soluble and insoluble fiber, as well as functional properties, such as water- and oil-holding capacity. Gorinstein et al. (2001) found that the peels of oranges, lemons, and grapefruits had higher total dietary fiber, soluble fiber, and insoluble fiber than these fruits without peel, on both fresh and dry weight bases. Besides these possible functional advantages, citrus fiber can provide health benefits. It has been reported that consuming a fiber-rich diet is associated with decreased incidence of atherosclerosis, hemorrhoids, and colon cancer (Rodriguez et al., 2006). Additionally, the antioxidant potential due to the presence of flavonoids, flavonols and polymethoxyflavones (PMFs) in citrus peel contributes functional and health benefits (Manthey and Grohmann, 1996; Bocco et al., 1998; Gorinstein et al., 2001). In most of those investigations, the citrus peels obtained for the

experiments were fresh rather than processed and no study has investigated the presence of these flavonoids in mass-produced citrus by-products.

Therefore, the objective of this research was to provide information about the identity and quantity of flavonoids found in mass-produced citrus fiber by reverse-phase HPLC and provide information on the effect of two types of processing procedures (regular washing and hot washing of peels) of citrus fiber on the TPC, TFC, TCC, and the antioxidant potential of such powder.

## MATERIALS AND METHODS

### Materials

Folin-Ciocalteu's phenol reagent was obtained from MP Biomedicals (Santa Ana, Calif., U.S.A.). Aluminum chloride anhydrous ( $\text{AlCl}_3$ ), potassium acetate ( $\text{CH}_3\text{COOK}$ ), sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), HPLC-grade water, HPLC-grade acetonitrile, HPLC-grade methanol, and glacial m-phosphoric acid were obtained from Fisher Scientific (Waltham, Mass., U.S.A.). Quercetin, kaempferol, and  $\beta$ -carotenoid were obtained (95% >purity by HPLC) from Sigma-Aldrich® Co. (St. Louis, Mo., U.S.A.). Gallic acid was obtained from Fluka® Analytical (St. Louis, Mo., U.S.A.). An ORAC antioxidant assay kit was obtained from Zen-Bio, Inc. (Research Triangle Park, N.C., U.S.A.). Nobiletin, sinensetin, tangeretin, and heptamethoxyflavone were kindly provided by Dr. John Manthey, U.S. Horticultural Research Laboratory, United States Department of Agriculture (USDA), Fort Pierce, Florida.

### Citrus Fiber

Sources of the fiber used in this study are albedo and flavedo parts of the orange peel. CitraFiber™ products were kindly provided by Natural Citrus Products Corporation (Fort Pierce, Fla., U.S.A.). Since the CitraFiber™ is a patented, commercially mass produced product, hot and cold wash procedures were done by the company. Products were fine ground in powder form. Nutrition profile of the both citrus fibers were same and can be seen in Table 1.

Table 1. Nutritional facts about citrus fiber CitraFiber™

Total Pectin	9390 mg/100g
Protein	6.37 %
Total Sugars	1.7%
Total Dietary Fiber	82.7%
Soluble Fiber	23.4%
Insoluble Fiber	59.3%
Potassium	453 mg/100g
Sodium	210 mg/100g
Calcium	78 mg/100g
Vitamin A (Beta Carotene)	117 IU/100g
Vitamin C	0.91 mg/100g

Source: Natural Citrus Products

### Standard Preparation

Stock solutions of quercetin (50 µg/ mL), kaempferol (120 µg/ mL), sinensetin (1060 µg/ mL), nobiletin (540 µg/ mL), heptamethoxyflavone (1900 µg/ mL), and tangeretin (820 µg/ mL) were prepared by dissolving these compounds individually in HPLC-grade methanol. The proper amounts of each standard and cocktail of standards were made by taking appropriate amounts from each standard and filtering them with a 0.45 µm filter before using them for the HPLC procedure.

### Aqueous Acetone Extraction

The methodology of Rodriguez-Saona and Wrolstad (2001) was used with modifications for this study. Ten g of sample was weighed into a beaker. Then, 40 mL of acetone (1:4 ratio of w/v) was measured and placed into the beaker and blended. Using a Buchner funnel, vacuum flask, and Whatman® no. 1 filter paper, the slurry was filtered. While keeping the filtered solution in the dark and under refrigeration, the filtered slurry was placed in a beaker, and 40 mL of 70/30 (v/v) acetone/acidified (0.01% HCl) water solution was added. After sonicating for one hour, the slurry was filtered again with a Buchner funnel, vacuum flask, and Whatman® no. 1 filter paper. This procedure was repeated two more times until a faint yellow color was observed. The collected filtrates were combined, and the liquid extract was placed into a 300 mL round-bottom flask. The acetone and water in the extract were completely evaporated using a rotary evaporator at 50 °C. The residue was dissolved in 10 mL of methanol.

This extract was used for the TPC, TFC, and ORAC values as well as HPLC analysis. The methanol extract was filtered with a 0.45 µm filter before being analyzed by HPLC. The extract was kept in the freezer at -18 °C during the experiment.

### HPLC Analysis

A Varian ProStar model 410 AutoSampler equipped with Varian 210-218 pumps and a Varian 335 UV detector was used for this study. Separation was achieved by using an Eclipse XDB C<sub>18</sub> 5 µm (4.6 × 250 mm) column (Agilent Technologies, Santa Clara, Calif., U.S.A.). The mobile phases consisting of 0.1% phosphoric acid in water (A) and acetonitrile (B) were used in a gradient program as follows: 100% (A) for the first 3 min, then 45% (A) for the next 9 min, and finally 5% (A) for the next 13 min. The mobile phase flow rate was 1 mL/min. The column temperature was kept at 30 °C. The injection volume was 20 µL. The chromatographic peaks were identified based on their retention times and compared with the retention times of the authentic standards. Flavonoid levels in the sample were calculated based on the external standard curve of the chosen flavonol and polymethoxyflavones (PMFs) standards.

### Determination of Total Polyphenol Content

To determine TPC, 0.1 mL of sample extracts were mixed with 2.8 mL of deionized water, 2 mL of 2% sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), and 0.1 mL of 50% Folin-Ciocalteu reagent. After the solution was incubated at room temperature for

30 min, the reaction mixture absorbance was measured at 750 nm. A deionized water blank was used for zeroing the spectrophotometer. Gallic acid was chosen as a standard, and a six-point standard curve was prepared (0-60 mg/L). The total polyphenol content of the citrus fiber was expressed as gallic acid equivalents (mg GA/g) for dry weight (Lin and Tang, 2007).

#### **Determination of Total Flavonoid Content**

To determine TFC, 0.5 mL sample extracts were mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride ( $\text{AlCl}_3$ ), 0.1 mL of 1M potassium acetate ( $\text{CH}_3\text{COOK}$ ), and 2.8 mL of deionized water. After the solution was incubated at room temperature for 30 min, the reaction mixture absorbance was measured at 415 nm. A deionized water blank was used for zeroing the spectrophotometer. Quercetin was chosen as a standard, and a seven-point standard curve was prepared (0-100 mg/L). The total flavonoid content of the citrus fiber was expressed as quercetin equivalents (mg QUE/g) for dry weight (Lin and Tang, 2007).

#### **Determination of Total Carotenoid Content**

A slurry of 2.5 g of citrus fiber and 25 mL of n-hexane-acetone-ethanol (v/v/v: 50:25:25) was placed on a shaker for 10 min at 200 rpm at room temperature. Then, it was centrifuged at 4500 g for 15 min at 8 °C. The supernatant was collected and made to a volume of 25 mL with hexane. Absorbance was measured at 450 nm.  $\beta$ -carotene was chosen as a standard, and a seven-point standard curve was prepared (0-100 mg/L). The total carotenoid content was expressed as beta-carotene equivalents (mg  $\beta$ -C/g) of dry weight. This methodology was adopted from Lee (2001), with some modifications.

#### **Oxygen Radical Absorbance Capacity Assay**

For this study, instructions from the Zen-Bio kit (Zen-Bio, Inc., Research Triangle Park, N.C., U.S.A.) were followed. A 96-well microplate was used for analysis. The plate reader incubation chamber was set to 37 °C (BioTek® Instruments, Inc., Synergy™ HT, Winooski, Vt., U.S.A.); excitation wavelength was set to 485 nm; and emission wavelength was set to 530 nm. After

preparation of the working solution and standard solutions, 150  $\mu\text{L}$  of the working solution was added to each well followed by 25  $\mu\text{L}$  of each sample, standard, or blank. Finally, 25  $\mu\text{L}$  of the AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) solution was added to each well and read over 30 min. Trolox was used as the standard, and a five-point (0-50  $\mu\text{M}$ ) standard curve was prepared. The ORAC values of the samples were calculated based on Davalos et al. (2004) and were expressed as  $\mu\text{mole Trolox/g}$ .

#### **Statistical Analysis**

Three replications of citrus fiber were evaluated for the TPC, TFC, TCC, and ORAC values. Data was analyzed by analysis of variance (ANOVA) using the general linear model (GLM) procedure of the SAS® Institute Inc. (2011). Means were separated by least significant difference (LSD) when significant ( $P < 0.05$ ) treatment effects were found.

## **RESULTS AND DISCUSSION**

Figure 1 shows the chromatogram of regular-washed citrus fiber. There were two groups of flavonoids found in the citrus samples. The first group was flavonols, and the second group was polymethoxyflavones. There was good separation of four major PMFs and flavonols, which eluted after 10 min. While glycosylated flavonones with an elution window of 8-10 min were also detected based on information obtained from running pure standards of glycosylated flavanones (naringin, hesperidin and isosakuranetinrutinoside), they were insufficiently separated for proper identification or quantitation. The method used in this study provides a fast analysis time for identification of flavonols and PMFs. The quantification of flavonols and polymethoxyflavones were carried out using external standard curves. The results are shown in Table 2.

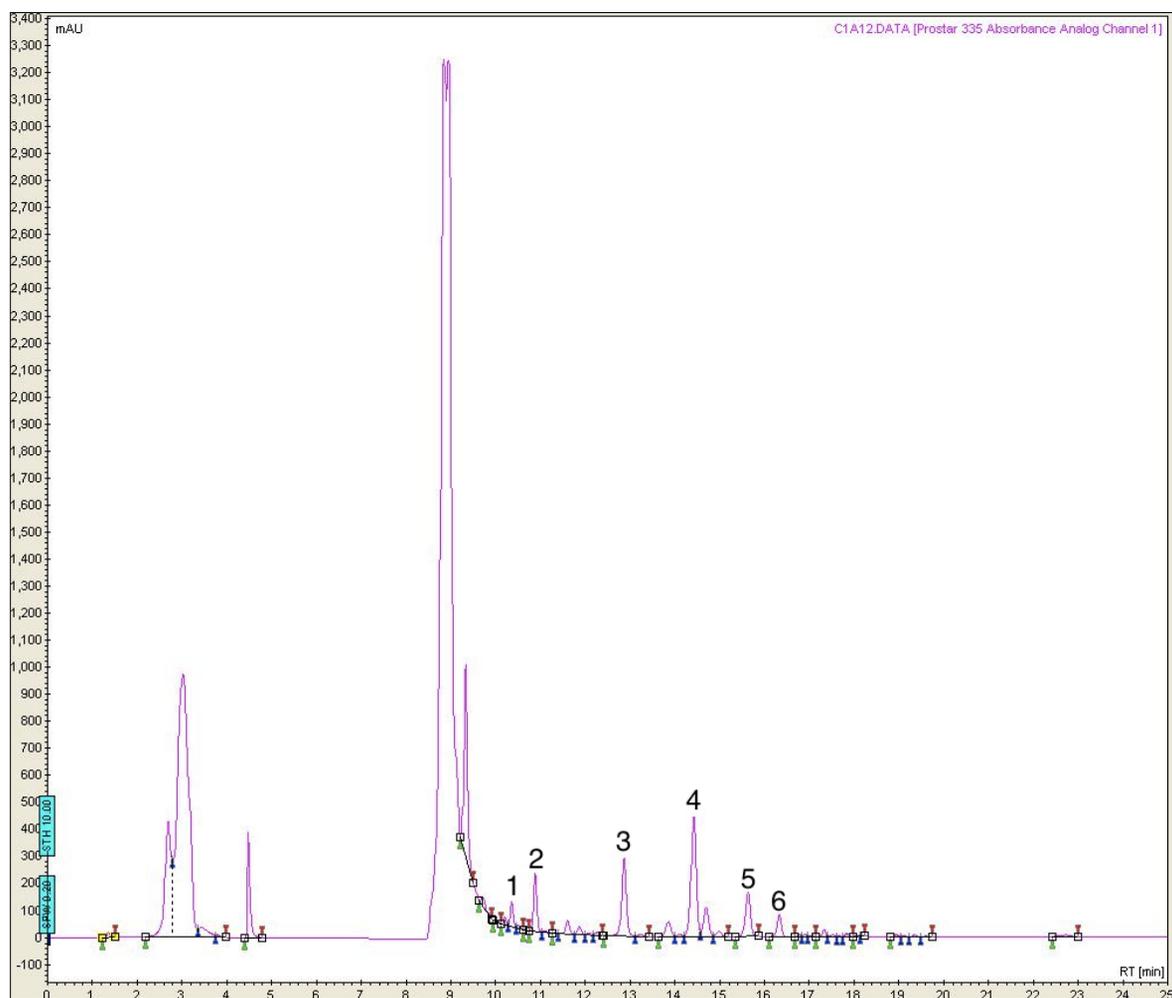


Figure 1. Reverse-Phase HPLC Chromatogram of Flavonols and Polymethoxyflavones Extracted from Regular-Washed Citrus Fiber

Peak identifications: 1 - quercetin (QUE), 2 - kaempferol (KAE), 3 - sinensetin (SIN), 4 - nobiletin (NOB), 5 - heptamethoxyflavone (HMF) and 6 - tangeretin (TAN).

Table 2. Flavonols and Polymethoxylated Flavones Concentration in Regular-Washed Citrus Fiber from Florida

Compound	Quantity (mg/g, dw)
Flavonols	
Quercetin	0.0276 ± 0.0107
Kaempferol	1.0945 ± 0.439
Polymethoxylated Flavones	
Sinensetin	1.9608 ± 0.958
Nobiletin	3.3304 ± 1.621
Heptamethoxyflavone	1.2155 ± 0.599
Tangeretin	0.5005 ± 0.251

Each value in the Table is represented as mean ± standard deviation (n=3).

Nobiletin had the highest concentration of all the flavones, followed by sinensetin, then heptamethoxyflavone. Similar results were obtained by Green et al. (2007). They found that Navel orange peels from the Jamaican cultivars had 3.8 mg/g nobiletin, 3.6 mg/g sinensetin and 2.6 mg/g heptamethoxyflavone. They also found that the majority of the Mexican citrus cultivars had nobiletin as the highest PMF in the citrus peels and nobiletin concentration for sweet orange peels type 1 and type 2 were 5.67 mg/g, 3.96 mg/g, respectively. The presence of flavonoids is influenced by the type of cultivar, as well as by the different tissues (albedo, flavedo,

juice vesicles) of citrus fruits (Nogata et al., 2006). Our findings were within the range of the results reported in earlier studies that focused on the identification and quantification of flavanone glycosides and PMFs (Manthey and Grohmann, 1996; Bocco et al., 1998; Green et al., 2007; Sun et al., 2010). A few studies also found the presence of flavonols, particularly quercetin and kaempferol, in citrus cultivars. Kawai et al. (1999) found quercetin in trace amounts in *Citrus paradisi* (grapefruit) juice extract, while kaempferol was found in trace amounts in *Citrus aurantium* (sour orange) juice extract. Also, Ross et al. (2000); Vanamala et al. (2006) reported the presence of quercetin in commercially available grapefruit juices. Wang et al. (2008) investigated the presence of flavonols in eight citrus species in Taiwan. They found trace amounts of quercetin and kaempferol in the peels of all citrus cultivars. Recently, Shalaby et al. (2011) identified the presence of kaempferol in *Citrus aurantifolia* (key lime) leaves, while quercetin was identified in both the leaves and peel of *Citrus sinensis* (sweet orange). Quercetin has shown some antiviral activity (Kaul et al., 1985; Tripoli et al., 2007). Also, it has been reported that polymethoxylated flavones provide antibacterial activity (Tripoli et al., 2007).

Table 3 shows the comparison of citrus fiber type on the presence of the TPC, TFC, TCC, and ORAC values. The results show that the total polyphenol content of citrus fibers significantly ( $P < 0.05$ ) differ between hot-washed and regular-washed citrus fibers. Total polyphenol content, total flavonoid content, total carotenoid content and antioxidant capacity of citrus fruits can be influenced by many factors, such as season, soil

quality, altitude, type of cultivar, processing procedure and whether it is a fresh or a dry product. Lutz et al. (2015) reported that total phenolic content and antioxidant capacity of fresh fruits were higher than dry counterpart. Ghasemi et al. (2009) reported similar results for the TFC for 13 cultivars of citrus peel including sweet orange, bitter orange, lemon etc., but they reported much higher total polyphenol contents (90.3-396.8 mg GAE/g) than our study found. Sankalpa et al. (2017) reported that processing method influenced the TFC, TPC, TCC and radical scavenging activity of sweet orange peel powder. There was no statistical difference ( $P > 0.05$ ) in the total flavonoid content and the total carotenoid content between hot-washed citrus fibers and regular-washed citrus fiber, which as shown in Table 3. Wang et al. (2008) reported the highest total carotenoid content in *Citrus reticulata* Blanco (tangerine) peel with  $2.04 \pm 0.036$  mg/g, while *Citrus sinensis* L. Osbeck (orange) peel had  $0.445 \pm 0.008$  mg/g, and *Citrus limon* L. Bur peel had  $0.110 \pm 0.001$  mg/g. While TFC and TCC were not affected by the processing method, the ORAC values were affected. Hot-washed samples had lower ORAC values than regular-washed samples. Li et al. (2006) reported that frozen lemon peel made from fresh lemons had significantly higher total polyphenol content than oven-dried lemon peel for dried bases. They suggested that a long drying time or temperature might cause a decrease in the total polyphenol content. Second, the use of hot water for citrus fiber preparation might lead to a reduction in the antioxidant capabilities of some of the polyphenols and flavonoids, resulting in much lower ORAC values.

Table 3. Effect of Extraction Methodology and Type of Citrus Fiber on Total Polyphenol Count, Total Flavonoid Count, and Oxygen Radical Absorbance Capacity of Citrus Fiber

Citrus Samples	TPC (mg GAE/g, dw)	TFC (mg QUEE/g, dw)	TCC (mg $\beta$ -CE/g, dw)	ORAC Value ( $\mu$ Mole Trolox/g, dw)
C1	$9.959 \pm 1.31^a$	$9.621 \pm 0.38$	$0.176 \pm 0.0060$	$27.834 \pm 6.86^a$
C2	$7.959 \pm 0.79^b$	$9.413 \pm 0.97$	$0.168 \pm 0.0095$	$15.589 \pm 4.53^b$

<sup>a, b</sup> Different letters in the same column indicate significant difference ( $P < 0.05$ ) analyzed by t-tests (least significant difference).

Each value in the Table is represented as mean  $\pm$  standard deviation ( $n = 3$ ).

C1: represents regular-washed samples.

C2: represents hot-washed sample

**CONCLUSION**

The high-pressure liquid chromatography technique used in this study provided fast results and good separation for polymethoxyflavones and flavonols in citrus fiber. Nobiletin, sinensetin, heptamethoxyflavone, tangeretin, quercetin, and kaempferol were identified. While quercetin was found in trace amounts, polymethoxyflavones were found in much higher quantities in the citrus fiber. Processing, i.e. hot-washing or regular-washing, of citrus fibers did not influence the TFC or the TCC. However, the regular-washed citrus samples had a higher total phenolic content and antioxidant capacity than the hot-washed citrus fibers. Results of our study also indicated that citrus fibers used in this study had relatively lower flavonoid concentration, TPC, TFC and ORAC values than what was found in the literature with fresh citrus peels.

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