Food Chemistry 171 (2015) 356–363

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Drying effect on flavonoid composition and antioxidant activity of immature kumquat

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ARTICLE INFO

Article history:
Received 4 May 2014
Received in revised form 23 August 2014
Accepted 29 August 2014
Available online 10 September 2014

Keywords:
Immature kumquat
Flavonoid composition
Antioxidant activity
Drying treatment

ABSTRACT

A seven flavonoids in hot water extract of immature kumquat (*Citrus japonica* var. margarita) were identified and quantified (mg/100 g fresh fruit): 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP, 285.9 ± 2.9 mg/100 g), acacetin 8-*C*-neohesperidoside (margaritene, 136.2 ± 2.6 mg/100 g), acacetin 6-*C*-neohesperidoside (isomargaritene, 119.1 ± 1.8 mg/100 g), fortunellin (acacetin 7-*O*-neohesperidoside, 28.5 ± 0.7 mg/100 g), apigenin 8-*C*-neohesperidoside (16.9 ± 0.1 mg/100 g), poncirin (isosakuranetin 7-*O*-neohesperidoside, 5.1 ± 0.1 mg/100 g), and rhoifolin (apigenin 7-*O*-neohesperidoside, 2.0 ± 0.1 mg/100 g). When immature kumquat was dried at 110 and 130 °C for 0.5 h (h), the antioxidant activity, total phenolic content and identified flavonoids increased. The UV absorbance of browning products of immature kumquat dried at 130 °C for 1.5 h increased dramatically, while the identified flavonoids decreased. Therefore, it was concluded that drying below 130 °C for 1.0 h, could release phenolic compounds, which resulted in the increasing antioxidant activity. Drying at 130 °C for 1.5 h, it might be due to the effect of formed browning products.

1. Introduction

Kumquat (*Citrus japonica* var. margarita) is a small size fruits and used to be candied or preserved whole in the sugar syrup. Dried whole kumquat is used to cure inflammatory syndrome of the respiratory tract (Chiu & Chang, 1998). Few studies have reported the flavonoid composition of kumquat. Eight flavonoids, included eriocitrin, narirutin, hesperidin, neohesperidin, luteolin, neoponcirin, poncirin, and kaempferol, in methanol/DMSO extract of kumquat have been reported and quantified (Kawail, Tomono, Katase, Ogawa, & Yano, 1999). However, only poncirin, didymin, isorhoifolin, hesperidin, and narirutin in 80% methanol extract are quantified (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011). For qualitative analysis, Ogawa, Kawasaki, Omura, and Yoshida (2001) demonstrated that 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), acacetin 8-*C*-neohesperidoside (margaritene), acacetin 6-*C*-neohesperidoside (isomargaritene), fortunellin (acacetin 7-*O*-neohesperidoside) existed in kumquat. However, DGPP and rutinoside derivatives of acacetin, instead of neohesperidoside derivatives of acacetin, are found in various solvent extracts (Sadek, Makris, & Kefalas, 2009). In another study, only DGPP, poncirin, narirutin, rutin, and apigenin 8-*C*-rutinoside are observed (Jayaprakasa, Murthy, Etlinger, Mantur, & Patil, 2012). Thus, the reported flavonoid compositions in kumquat are quite different, except for DGPP and poncirin. The quantitative data of these flavonoids in kumquat are still lacking. In addition, no study about the immature kumquat has been reported.

It is well known that dried citrus peel is used as traditional Chinese medicine (Choi et al., 2011; Zang, 2005). Studies about the effect of heat treatment on phenolic compounds and antioxidant activity of citrus peel have been reported (Choi et al., 2011; Ho & Lin, 2008; Jeong et al., 2004; Xu, Ye, Chen, & Liu, 2007). It was suggested that high temperature drying might be used as an effective method to release bound phenolic compounds from citrus and increase their antioxidant activity (Choi et al., 2011; Gil-Izquierdo, Gil, & Ferreres, 2002; Xu et al., 2007). In our previous study, the antioxidant activity and total phenolic content of immature calamondin increase significantly after heat treatment at 150 °C for 1.5 h (Lou, Lin, Hsu, Chiu, & Ho, 2014). It concluded that high temperatures could enhance the extraction efficacy of phenolic content; however, some flavonoids such as DGPP could be degraded. It has also been demonstrated that proper heat

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http://dx.doi.org/10.1016/j.foodchem.2014.08.119
0308-8146/

treatment could enhance the antioxidant and anti-inflammatory activities of kumquat peel (Lin, Hung, & Ho, 2008). Therefore, the purpose of this study was to investigate the flavonoid composition of immature kumquat. In addition, the changes in flavonoid composition as affected by drying conditions were evaluated. A proper drying condition to enhance the antioxidant activity of kumquat was also proposed.

2. Materials and methods

2.1. Materials

Kumquat (*C. japonica* var. margarita) was collected from a kumquat estate in the Jao-Si region, Ilan, Taiwan in November 2010. The collected kumquat, after flowering for 120 days, with whole green appearance was sorted as immature kumquat and had average weight of 17.48 ± 1.35 g. After washing, kumquat was cut into ca. 0.5 cm slices. Then, they were lyophilised for 48 h. Prior to extraction, the kumquat slices were pulverised in a blender and passed through a 60 mesh sieve. The obtained powders of immature kumquat were stored in a suitable brown bottle with screw cap at −18 °C.

2.2. Chemicals

Methanol, ethanol, ethyl acetate, and acetonitrile, were LC grade from Merck Chemical Co. (Darmstadt, Germany). Acetic acid, Na_2CO_3, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), disodium fluorescein (FL), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were analytical grade. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97% (Trolox), standards of flavonoids, including diosmin, diosmetin, hesperetin, hesperidin, kaempferol, luteolin, naringin, naringenin, neohesperidin, nobiletin, quercetin, rutin, tangeretin, sinensetin, neoeriocitrin, fortunellin, poncirin, and rhoifolin, and standards of phenolic acids, including caffeic acid, syringic acid, gentisic acid, ferulic acid, ellagic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, vanillic acid, protocatechuic, acid and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3′,5′-Di-*C*-β-glucopyranosylphloretin (DGPP) was obtained from immature calamondin according to Lou, Yu, and Ho (2012). Acacetin 8-*C*-neohesperidoside (margaritene), acacetin 6-*C*-neohesperidoside (isomargaritene) and apigenin 8-*C*-neohesperidoside were obtained from hot water extract of immature kumquat in our laboratory. The extract was separated by a semi-preparative HPLC and the compounds were collected. Then, they were subjected to LC/MS^n for identification. The estimated purities for margaritene, isomargaritene and apigenin 8-*C*-neohesperidoside were 90.4%, 97.9% and 72.2%, respectively, based on area of HPLC chromatogram by UV 280 nm measurement. After lyophilization, the residues were redissolved in deionised water and stored at −18 °C for further use.

2.3. Heat treatment

The sliced immature kumquats were subjected to hot air drying in an oven at 110 and 130 °C for 0.5, 1.0, 1.5 and 2.0 h, separately, and 150 °C for 1.5 h. The obtained dried sliced immature kumquats were then lyophilised for 48 h. Prior to extraction, the lyophilised immature kumquats were pulverised in a blender and passed through a 60 mesh sieve. The obtained powders of hot air dried immature kumquat were stored in a suitable brown bottle, filled with nitrogen, with screw cap at −18 °C.

2.4. Preparation of hot water extracts

Three grams of dried and powdered immature kumquats were extracted with 60 mL deionised hot water (90 °C) for 1 h in a shaking water bath at the same temperature. The shaking rate was 100 rpm. The extract was filtered with a Whatman No. 1 filter paper. The obtained residue was extracted by the same procedure two more times. Three resulting filtrates were transferred into a 250 mL flask and dried by a rotary vacuum evaporator at 40 °C. To dissolve the filtrate, a suitable volume of deionised water was added to the flask for the extract. The obtained solutions were poured into brown bottles with screw cap and stored at −18 °C until further use. Triplicate determinations ($n = 3$) were carried out for the study.

2.5. Identification of flavonoids by HPLC-UV-ESI/MS analysis

A Thermo Fisher SpectraSystem P4000 HPLC coupled with a UV detector (Shimadzu SPD 10 Avp, Japan) and a Thermo Fisher Scientific LCQ-Fleet mass spectrometer were used for the identification of flavonoid composition. The separation was performed on a reverse phase column (Discovery RP-C18, 250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) using a gradient with acetonitrile as solvent A and deionised water as solvent B (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011). The gradient was carried out as 0–15 min, 95–80% B; 15–20 min, 80–70% B; 20–35 min, 70–0% B; 35–40 min, 0–95% B; 40–50 min, 95% B for equilibrium. The flow rate was 1 mL/min. The chromatogram was recorded at 280 nm. Ion trap mass spectrometer was equipped with an electrospray ionisation (ESI) source. Mass spectra were obtained at positive and negative ion modes. The source parameters were as follows: ESI source voltage of 5 kV, capillary temperature 300 °C, Sheath gas 40 arbitrary. The tube lens voltage was 0 for positive ion mode and −25 V for negative ion mode. Full scan MS was measured from m/z 160 to 1000. Collision-induced fragmentation experiments were performed using helium as the collision gas. The instrument operated under Xcalibur version 2.5 delivered by Thermo Fisher.

2.6. HPLC analysis of flavonoid composition

The hot water extracts of immature kumquat were subjected to a HPLC analysis (Shimadzu LC-10AT) with a Discovery RP-C18 column (250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA). The same separation method (Barreca et al., 2011) as described in paragraph 2.5 was used. Photodiode array (PDA) detection was performed between 220 and 350 nm, with a resolution of 2 nm. Seven flavonoids used as standards were available in our laboratory as follows: DGPP, margaritene, isomargaritene, apigenin 8-*C*-neohesperidoside, fortunellin, poncirin, and rhoifolin. The flavonoid compounds were quantified from peak area at 280 nm by an external standard method, using calibration curves. Their concentrations were expressed as milligram per 100 g dry weight.

2.7. Determination of total phenolic content

A hundred microlitres of hot water extract from samples, or standard solution, were mixed with 100 μL of Folin–Ciocalteu's phenol reagent for 3 min (Taga, Miller, & Pratt, 1984). The mixture was added to 1 mL of 20% Na_2CO_3 solution and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 750 nm against the blank. The standard curve was determined with gallic acid, and the total phenolic content was expressed as mg gallic acid equivalent (GAE) per 100 g dry extract using the standard curve. All samples were analysed in triplicate.

2.8. DPPH radical scavenging activity

The DPPH radical scavenging activity of immature kumquat extracts was measured according to a slightly modified method

of Yamaguchi, Takamura, Matoba, and Terao (1998). After 0.5 mL of immature kumquat extract was mixed with 0.5 mL of 0.5 mM DPPH in methanol for 30 min, the mixture was subjected to HPLC analysis with reverse phase column (Thermo ODS-2 Hypersil, 250 × 4.6 mm, 5 μm) under photodiode array (PDA) detection at 517 nm. The mobile phase was methanol/water (7/3, v/v) and the flow rate was 1 mL/min. The change in peak area of DPPH was determined after the reaction. Radical scavenging activity was expressed as percent inhibition and was calculated using the following formula:

%DPPH radical scavenging activity

$$= (1 - \text{peak area in sample/peak area in blank}) \times 100.$$

2.9. Oxygen radical absorbance capacity (ORAC) assay

The reaction was carried out in 75 mM phosphate buffer (pH 7.4) in cuvettes (Ou, Hampsch-Woodill, & Prior, 2001). Fifty microlitres of plant extract solution and 50 μL of disodium fluorescein (70 nM final concentration) were mixed in a cuvette and preincubated for 15 min at 37 °C. Twenty-five microlitres of APPH solution (221 mM final concentration) was then added, and fluorescence was recorded for 70 min at excitation and emission wavelengths of 485 and 520 nm every 5 min. A blank sample containing phosphate buffer in the reaction mix was measured. Five calibration solutions of Trolox (10, 20, 30, 40, and 50 μM final concentrations) were also tested to establish a standard curve. All samples were analysed in triplicate. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and ORAC values were expressed as mmol Trolox equivalents/g plant extract or fractions using the standard curve established previously.

2.10. Determination of absorbance at UV_{420nm}

According to Kim and Lee's method (2009) with minor modification, the hot water extracts of immature kumquat by different drying condition were diluted with distiled water to 1:19, v/v. Then, absorbance of 200 μL resulting solution was determined by ELISA reader at 420 nm.

2.11. Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.05$), using SAS (SAS Inst., Cary, NC, USA).

3. Results and discussion

3.1. Flavonoid composition of immature kumquat

Chromatograms of the HPLC coupled with PDA detector were shown in Fig. 1. Fifteen major compounds were numbered as **1**–**15** and identified base on retention time, UV spectra and MS2 data (Table 1).

Compound **1** (λ_{max} = 332, 270 nm) showed pseudomolecular ions of *m*/*z* 593 ($[M-H]^-$) and *m*/*z* 595 ($[M+H]^+$. The negative ion mode of MS2 spectra revealed 575 $[(M-H)-18]^-$, 503 $[(M-H)-90]^-$, 473 $[(M-H)-120]^-$, 383 $[(M-H)-210]^-$ (aglycone + 113) and 353 $[(M-H)-240]^-$ (aglycone + 83). The UV spectrum and MS data coincided with di-*C*-hexosyl flavones. The ions at *m*/*z* 383 and *m*/*z* 353 indicated that the aglycone was apigenin. Therefore, it was

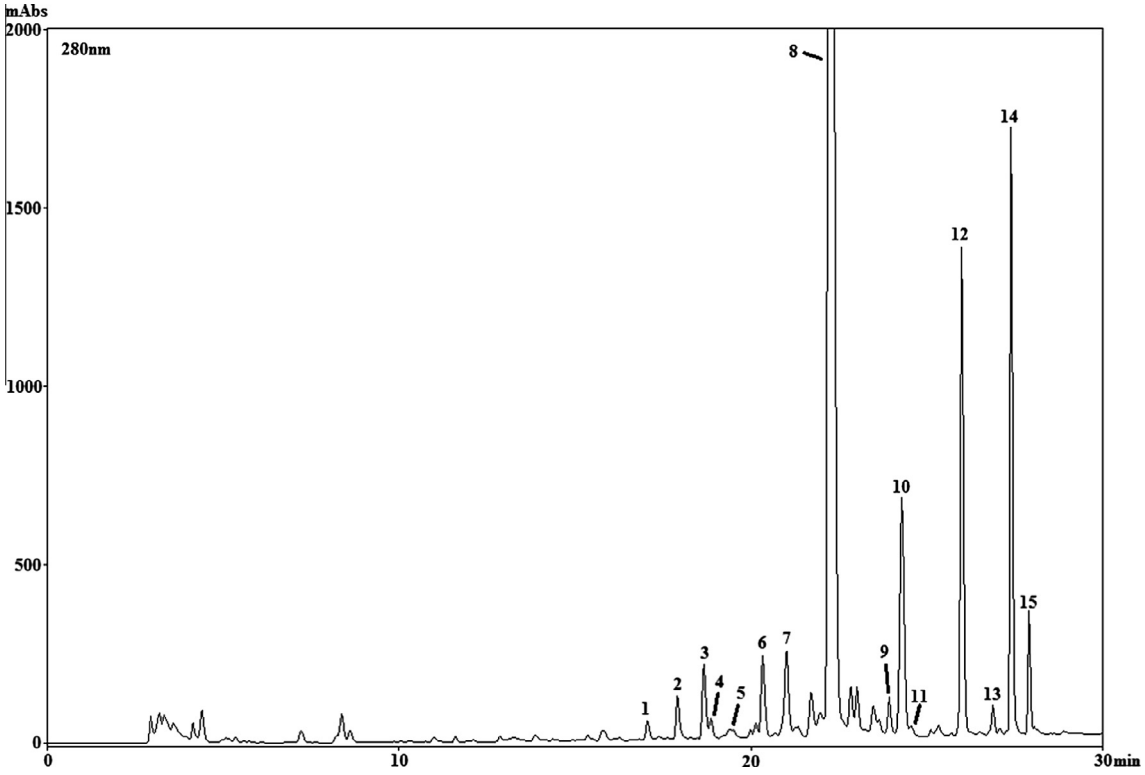

Fig. 1. HPLC profiles of hot water extracts from immature kumquat. (Peak number: (1) apigenin-6,8-di-*C*-glucoside, (2) flavanone di-*C*-pentose–hexose, (3) flavanone di-*C*-pentose–hexose, (4) luteolin-8-*C*-neohesperidoside, (5) luteolin 6-*C*-neohesperidoside, (6) flavanone di-*C*-pentose–hexose, (7) apigenin-8-*C*-neohesperidoside (8) 3′, 5′-di-*C*-β-glucopyranosylphloretin (DGPP) (9) apigenin 7-*O*-neohesperidoside (rhoifolin), (10) acacetin 8-*C*-neohesperidoside (margaritene), (11) unknown, (12) acacetin 6-*C*-neohesperidoside (isomargaritene), (13) di-*C*-hexoxyl derivative, (14) acacetin 7-*O*-neohesperidoside (fortunellin), (15) isosakuranetin 7-*O*-neohesperidoside (poncirin)).

Table 1
Retention time, maximum UV spectra, MS fragment ions of compounds in hot water extract from immature kumquat.

Peak	T_R (min)	λ_{max} (nm)	$[M-H]^-$	MS^2 fragment ions on $[M-H]^-$ m/z (%) [a]	$[M+H]^+$	MS^2 fragment ions on $[M+H]^+$ m/z (%)
1	16.93	270, 332	593	575 [M−H-18]$^-$ (4), 503 [M−H-90]$^-$ (32), 473 [M−H-120]$^-$ (100), 383 [M−H-210]$^-$ (22), 353 [M−H-240]$^-$ (40)	595	577 [M+H-18]$^+$ (100), 559 [M+H-36]$^+$ (24), 529 [M+H-66]$^+$ (20), 475 [M+H-120]$^+$ (12), 457 [M+H-138]$^+$ (30)
2	17.77	285, 330(sh)	579	561 [M−H-18]$^-$ (15), 543 [M−H-36]$^-$ (1), 519 [M−H-60]$^-$ (4), 489 [M−H-90]$^-$ (20), 459 [M−H-120]$^-$ (100), 315 [M−H-264]$^-$ (1)	581	563 [M+H-18]$^+$ (100), 545 [M+H-36]$^+$ (4), 527 [M+H-54]$^+$ (7), 515 [M+H-66]$^+$ (4), 461 [M+H-120]$^+$ (20)
3	18.54	284, 331(sh)	579	561 [M−H-18]$^-$ (10), 543 [M−H-36]$^-$ (1), 519 [M−H-60]$^-$ (1), 489 [M−H-90]$^-$ (20), 471 [M−H-108]$^-$ (1), 459 [M−H-120]$^-$ (100)	581	563 [M+H-18]$^+$ (100), 545 [M+H-36]$^+$ (4), 527 [M+H-54]$^+$ (7), 515 [M+H-66]$^+$ (4), 461 [M+H-120]$^+$ (20)
4	18.71	269, 349	593	575 [M−H-18]$^-$ (8), 473 [M−H-120]$^-$ (100), 447 [M−H-146]$^-$ (14), 429 [M−H-164]$^-$ (64), 357 [M−H-236]$^-$ (20), 327 [M−H-266]$^-$ (6), 309 [M−H-284]$^-$ (10), 285 [M−H-308]$^-$ (1)	595	577 [M+H-18]$^+$ (8), 449 [M+H-146]$^+$ (100), 431 [M+H-164]$^+$ (18), 395 [M+H-200]$^+$ (1), 383 [M+H-212]$^+$ (5), 353 [M+H-242]$^+$ (1), 329 [M+H-266]$^+$ (1),
5	19.25	269, 335	593	575 [M−H-18]$^-$ (2), 473 [M−H-120]$^-$ (100), 449 [M−H-144]$^-$ (8), 429 [M−H-164]$^-$ (38), 357 [M−H-236]$^-$ (42), 327 [M−H-266]$^-$ (42), 309 [M−H-284]$^-$ (2), 285 [M−H-308]$^-$ (1)	595	577 [M+H-18]$^+$ (2), 449 [M+H-146]$^+$ (100), 431 [M+H-164]$^+$ (18), 329 [M+H-266]$^+$ (10), 287 [M+H-308]$^+$ (4)
6	20.20	285, 331(sh)	579	561 [M−H-18]$^-$ (10), 543 [M−H-36]$^-$ (1), 519 [M−H-60]$^-$ (1), 489 [M−H-90]$^-$ (18), 459 [M−H-120]$^-$ (100), 411 [M−H-168]$^-$ (1),	581	563 [M+H-18]$^+$ (100), 545 [M+H-36]$^+$ (18), 527 [M+H-54]$^+$ (20), 561 [M+H-120]$^+$ (18), 419 [M+H-162]$^+$ (4)
7	20.86	269, 339	577	559 [M−H-18]$^-$ (1), 457 [M−H-120]$^-$ (18), 431 [M−H-146]$^-$ (4), 413 [M−H-164]$^-$ (100), 293 [M−H-284]$^-$ (38), 269 [M−H-308]$^-$ (1)	579	561 [M+H-18]$^+$ (1), 459 [M+H-120]$^+$ (1), 433 [M+H-146]$^+$ (100), 415 [M+H-164]$^+$ (18), 271 [M+H-308]$^+$ (4)
8	22.20	228, 285	597	579 [M−H-18]$^-$ (18), 561 [M−H-36]$^-$ (4), 507 [M−H-90]$^-$ (10), 489 [M−H-108]$^-$ (10), 477 [M−H-120]$^-$ (100), 459 [M−H-138]$^-$ (10)	599	581 [M+H-18]$^+$ (58), 563 [M+H-36]$^+$ (100), 545 [M+H-54]$^+$ (38), 461 [M+H-138]$^+$ (22)
9	23.82	267, 335	577	457 [M−H-120]$^-$ (4), 431 [M−H-146]$^-$ (5), 413 [M−H-164]$^-$ (6) 311 [M−H-266]$^-$ (3), 269 [M−H-308]$^-$ (100)	579	561 [M+H-18]$^+$ (18), 433 [M+H-146]$^+$ (38), 429 [M+H-150]$^+$ (2), 334 [M+H-245]$^+$ (1), 271 [M+H-308]$^+$ (100)
10	24.24	269, 334	591	573 [M−H-18]$^-$ (1), 471 [M−H-120]$^-$ (24), 445 [M−H-146]$^-$ (4), 427 [M−H-164]$^-$ (100), 367 [M−H-224]$^-$ (22), 307 [M−H-284]$^-$ (84)	593	575 [M+H-18]$^+$ (1), 473 [M+H-120]$^+$ (1), 447 [M+H-146]$^+$ (100), 429 [M+H-164]$^+$ (24), 285 [M+H-308]$^+$ (8)
11	24.51	267, 330	577	–[b]	579	561 [M+H-18]$^+$ (1), 489 [M+H-90]$^+$ (1), 459 [M+H-120]$^+$ (8), 447 [M+H-132]$^+$ (100), 411 [M+H-168]$^+$ (16)
12	25.92	270, 334	591	573 [M−H-18]$^-$ (2), 471 [M−H-120]$^-$ (36), 445 [M−H-146]$^-$ (2), 427 [M−H-164]$^-$ (100), 367 [M−H-224]$^-$ (84), 307 [M−H-284]$^-$ (88),	593	575 [M+H-18]$^+$ (18), 447 [M+H-146]$^+$ (100), 429 [M+H-164]$^+$ (22), 411 [M+H-182]$^+$ (10), 285 [M+H-308]$^+$ (10)
13	26.82	285, 330 (sh)	581	563 [M−H-18]$^-$ (20), 545 [M−H-36]$^-$ (1), 491 [M−H-90]$^-$ (16), 473 [M−H-108]$^-$ (20), 461 [M−H-120]$^-$ (100), 341 [M−H-240]$^-$ (44)	583	565 [M+H-18]$^+$ (100), 547 [M+H-36]$^+$ (50), 463 [M+H-120]$^+$ (38), 445 [M+H-138]$^+$ (4), 427 [M+H-156]$^+$ (50)
14	27.36	267, 331	591	575 [M−H-16]$^-$ (4), 471 [M−H-120]$^-$ (9), 427 [M−H-164]$^-$ (22), 355 [M−H-236]$^-$ (10), 337 [M−H-254]$^-$ (10), 307 [M−H-284]$^-$ (100),	593	575 [M+H-18]$^+$ (1), 447 [M+H-146]$^+$ (20), 413 [M+H-180]$^+$ (1), 285 [M+H-308]$^+$ (100), 270 [M+H-323]$^+$ (4)
15	27.84	283, 323	593	–[b]	595	448 [M+H-147]$^+$ (100), 429 [M+H-166]$^+$ (90), 351 [M+H-244]$^+$ (9), 286 [M+H-309]$^+$ (12),

[a] Values in brackets represent relative abundance, and main observed fragment. Other ions were found but they have not been included.
[b] Data not be obtained.

tentatively identified as apigenin-6,8-di-*C*-glucoside (Barreca et al., 2011; Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003; Roowi & Crozier, 2011), which has been reported in *Fortunella japonica* (Barreca et al., 2011; Cho et al., 2005; Kumamoto, Matsubara, Iizuka, Okamoto, & Yokoi, 1985), however, it has not been reported in kumquat previously.

Compounds **2** (λ_{max} = 330 (sh), 285 nm), 3 (λ_{max} = 331 (sh), 284 nm), and 6 (λ_{max} = 331 (sh), 285 nm) have the same pseudomolecular ions at m/z 579 $[M-H]^-$ and 581 $[M+H]^+$. They also showed the similar negative ion fragmentation pattern of MS2 spectra with m/z 561 [(M−H)-18]$^-$, 519 [(M−H)-60]$^-$, 489 [(M−H)-90]$^-$, and 459 [(M−H)-120]$^-$ (base peak). The presence of a fragment ion at m/z 489 [(M−H)-90]$^-$ and m/z 459 for [(M−H)-120]$^-$, and the absence of the aglycone ion, which is typical of di-*C*-glycosyl flavonoids (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2010; Barreca et al., 2011; Ferreres et al., 2003; Roowi & Crozier, 2011). The ion at m/z 519 [(M−H)-60]$^-$ characterises the presence of a pentose (Ferreres et al., 2003). The similar UV spectrum of these three compounds revealed that they were flavanones. Therefore, it was suggested that compounds **2**, **3**, and **6** were flavanone (286) with di-*C* linkage of pentose (132) and hexose (162).

Compounds **4** (λ_{max} = 349, 269 nm) and 5 (λ_{max} = 335, 269 nm) showed the UV spectrum coincided with the flavones. They have the same pseudomolecular ions at m/z 595 $[M+H]^+$ and 593 $[M-H]^-$. The similar pattern of fragmentation by MS2 was observed with m/z 575 [(M−H)-18]$^-$, 473 [(M−H)-120]$^-$ (base peak), 429 [(M−H)-164]$^-$, 357 [(M−H)-236]$^-$, 327 [(M−H)-266]$^-$, 309 [(M−H)-284]$^-$, and 285 [(M−H)-308]$^-$. The presence of the m/z 473 [(M−H)-120]$^-$ (base peak) in negative ion pattern of MS2 indicated *C*-glycoside, while no aglycone was observed. The fragment positive ion at m/z 449 [(M+H)-146]$^+$ revealed a loss of a rhamnosyl unit. An ion at m/z 285 [(M−H)-308]$^-$ and 287 [(M+H)-308]$^+$ provides evidence that a disaccharide moiety (146 + 162) existed and the molecular ion of aglycone was m/z 286. The ion at m/z 473 [(M−H)-120]$^-$ indicated also that the disaccharide moiety attached to the aglycone is a neohesperidoside rather than a rutinoside (Barreca et al., 2011; Ma, Cuyckens, Van den Heuvel, & Claeys, 2001; Truchado, Vit, Ferreres, & Tomas-Barberan, 2011). All these data indicated that compounds **4** and **5** are flavone (286) 6-*C* or 8-*C*-neohesperidoside. Therefore, compounds **4** and **5** were tentatively identified as luteolin-8-*C*-neohesperidoside and luteolin 6-*C*-neohesperidoside, respectively, since the 8-*C*-glycosyl derivative eluted before its *C*-6 isomers in RP-HPLC system (Ferreres et al., 2003).

Compound **7** showed pseudomolecular ion at 577 $[M-H]^-$ and MS2 spectra with 457 [(M−H)-120]$^-$, 431 [(M−H)-146]$^-$, 413 [(M−H)-164]$^-$ (base peak), 293 [(M−H)-284]$^-$, and 269 [(M−H)-308]$^-$. The data indicated that a *C*-glycoside linkage disaccharide moiety existed, which contained rhamnose and hexose. It was

identified as neohesperidose. The *m*/*z* of aglycone is at 270 and the UV spectra revealed a flavone. Therefore, compound **7** was tentatively identified as apigenin-8-*C*-neohesperidoside after comparing with the data of *F. japonica* study by Barreca et al. (2011).

Compounds **8**, **9**, **14**, and **15** were identified by retention time, UV spectrum, MS, MS2 data in comparing with standard substances. Compound **8** was identified as 3′,5′-di-*C*-glucopyranosylphloretin (DGPP). Compound **9** is apigenin 7-*O*-neohesperidoside (rhoifolin). Compound **14** is acacetin 7-*O*-neohesperidoside (fortunellin). Compound **15** is isosakuranetin 7-*O*-neohesperidoside (poncirin).

Compounds **10** and **12** have the same pseudomolecular ion at *m*/*z* 591 $[M-H]^-$ and 593 $[M+H]^+$. The MS2 spectra yielded *m*/*z* 471 $[(M-H)-120]^-$, 445 $[(M-H)-146]^-$, and 285 $[(M+H)-308]^+$ for both compounds, which indicated neohesperidoside and the aglycone is 284 m.u. These coincided with the data of Barreca et al. (2011). It is concluded that compound **10** is acacetin 8-*C*-neohesperidoside (margaritene) and compound **12** is acacetin 6-*C*-neohesperidoside (isomargaritene).

Compounds **13** showed a pseudomolecular ion at *m*/*z* 581 $[M-H]^-$ and 583 $[M+H]^+$. The MS2 spectra yielded *m*/*z* 461 $[(M-H)-120]^-$ and 341 $[(M-H)-240]^-$, which indicated a di-*C*-hexoxyl group. The aglycone could be 258 m.u., while *m*/*z* 341 might be equal to *m*/*z* (aglycone + 83). The structure of compound **11** is still not clear. The structures of identified compounds of immature kumquat are shown in Fig. 1.

3.2. Quantitative evaluation

The three major flavonoids in hot water extract of immature kumquat were *C*-glycosyl dihydrochalcone and *C*-glycosyl methylated flavone, which were DGPP, margaritene, and isomargaritene. The levels of these three flavonoids were 285.9 ± 2.9, 136.2 ± 2.6, and 119.1 ± 1.8 mg/100 g fresh kumquat (Table 2) that were 48.2%, 22.9%, and 20.0% of the total identified flavonoids, respectively. Large quantity of DGPP has already been reported from *Fortunella* spp. (Barreca et al., 2011; Jayaprakasa et al., 2012; Kumamoto et al., 1985; Ogawa et al., 2001; Sadek et al., 2009), immature calamondin (Lou et al., 2012; Yu, Lou, & Ho, 2013), and also *Citrus microcarpa*, *Citrus medica* var. L, and *Citrus hystrix* (Roowi & Crozier, 2011). DGPP has been reported to have good tyrosinase inhibitory activity (Lou et al., 2012). Acacetin 8-*C* (margaritene) and 6-*C* (isomargaritene) neohesperidosides have been reported in kumquat (Ogawa et al., 2001) and also in *F. japonica* (Barreca et al., 2011; Cho et al., 2005; Kumamoto et al., 1985). However, acacetin 8-*C*-rutinoside and acacetin 6-*C*-rutinoside, instead of neohesperidoside, were reported in ethyl acetate and butanol extracts from kumquat by Sadek et al. (2009). In their study, all the related compounds were reported as rutinoside, which was identified based on retention time and MS spectra. These might lead to some uncertainty of the identified compounds.

Table 2
Flavonoid composition of immature kumquat extracted by hot water.

Flavonoids	Dry kumquat[g] (mg/100 g)	Fresh kumquat (mg/100 g)
3′,5′-Di-*C*-β-glucopyranosylphloretin	1979 ± 20[a]	285.9 ± 2.9[a]
Acacetin 8-*C*-neohesperidoside	942 ± 18[b]	136.2 ± 2.6[b]
Acacetin 6-*C*-neohesperidoside	824 ± 13[b]	119.1 ± 1.8[b]
Fortunellin	197 ± 5[c]	28.5 ± 0.7[c]
Apigenin 8-*C*-neohesperidoside	117 ± 1[d]	16.9 ± 0.1[d]
Poncirin	35 ± 1[e]	5.1 ± 0.1[e]
Rhoifolin	14 ± 1[f]	2.0 ± 0.1[f]
Total	4108 ± 30	593.7 ± 4.4

[a–f]Values (Mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).
[g]Lyophilised kumquat.

The contents of fortunellin (acacetin 7-*O*-neohesperidoside) and Apigenin-8-*C*-neohesperidoside were 28.5 ± 0.7 and 16.9 ± 0.1 mg/100 g fresh kumquat. Similar compound, acacetin 7-*O*-rutinoside, has been reported in kumquat (Sadek et al., 2009). Fortunellin has also been identified in juice of *F. Japonica* (Barreca et al., 2011). Poncirin (isosakuranetin 7-*O*-neohesperidosie) and rhoifolin (apigenin 7-*O*-neohesperidoside) were in minor quantity below 5 mg/100 g fresh kumquat.

The main disaccharide of the flavonoids in kumquat is neohesperidose, while glucose was found in DGPP. The content of *C*-glycoside flavonoids dominated in kumquat, especially DGPP. The biological activity of these *C*-glycoside compounds has not been investigated. However, the aglycones, phloretin and acacetin, exhibit broad spectrum of biological activities, such as antioxidant activity, anti-inflammatory effect and anticancer effect (Hsu, Kuo, & Lin, 2004; Liao, Houghton, & Hoult, 1999; Pan, Lai, Wang, & Ho, 2006; Rezk, Haenen, Van der Vijgh, & Bast, 2002; Shao et al., 2008).

3.3. Antioxidant activity of immature kumquat affected by drying

In our previous studies, the total phenolic and flavonoid contents of the calamondin extract increase when fruits are dried at 150 °C for 1.5 h before extraction, while no obvious changes are observed when drying temperatures are below 100 °C (Lou et al., 2014). Similar changes were also observed in kumquat in this study. We also found that the antioxidant activity of kumquat extract when kumquat was heated at 150 °C was much higher than when it was heated at 100 °C (Chao, 2011). Therefore, a study was carried out to elucidate the changes of antioxidant activity of extracts when kumquat was heated at 100–150 °C.

The DPPH free radical scavenging activity of hot water extracts from immature kumquat after drying at 110, 130 °C for 0.5, 1.0, 1.5, and 2.0 h, as well as at 150 °C for 1.5 h is shown in Table 3. The DPPH scavenging potency increased significantly after drying at 110 °C for 1.5 h and 130 °C for 0.5 h. The obvious increase of the DPPH scavenging potency was observed at 130 °C drying for over 1.5 h. The highest scavenging potency was shown at 150 °C for 1.5 h. The scavenging potency was enhanced from 11.5%/mg/mL for fresh kumquat to 34.6%/mg/mL for 150 °C drying. Fig. 2 (A) revealed the ORAC values for the hot water extracts of immature kumquat. The changes of ORAC were similar to the changes of DPPH. The ORAC increased after drying at temperature over 110 °C. Interestingly, the ORAC value also increased drastically at 130 and 150 °C for over 1.5 h, as the changes of DPPH.

Collectively, these results indicated that drying process could enhance the antioxidant activity of hot water extract from immature kumquat. The higher temperature or longer drying duration was performed, the higher antioxidant activity exhibited. For immature kumquat, the critical point to obtain higher antioxidant activity of the hot water extract was drying over 130 °C for over 1.5 h. It has been reported that the antioxidant activity of citrus peel extract increased as heating temperature increased (Jeong et al., 2004). In our previous study, we also observed that the antioxidant activity of hot water extract from immature calamondin peel could be enhanced by heating at 150 °C for 1.5 h (Lou et al., 2014).

3.4. Changes in flavonoid composition of immature kumquat during drying

The immature kumquat was dried at different temperatures and time durations, then extracted by hot water and subjected to HPLC analysis for the flavonoid composition. The changes in

Table 3
The DPPH radical scavenging potency and absorbance at UV_{420nm} of hot water extract from immature kumquat affected by drying process.[i]

Drying temperature (°C)	Drying time (hours)			
	0.5	1.0	1.5	2.0
Scavenging potency[f] *(%/mg/mL)*				
110	12.07 ± 0.89^{cde}	12.40 ± 0.42^{cde}	12.93 ± 0.67^{cd}	13.33 ± 0.25^{c}
130	12.90 ± 0.47^{cd}	12.88 ± 0.72^{cd}	17.85 ± 0.09^{b}	18.55 ± 0.09^{b}
150	–[h]	–	34.64 ± 0.91^{a}	–
Absorbance at UV^{g}_{420nm}				
110	0.471 ± 0.054^{e}	0.349 ± 0.051^{e}	0.619 ± 0.109^{cd}	0.861 ± 0.091^{c}
130	0.416 ± 0.038^{e}	0.606 ± 0.015^{d}	2.960 ± 0.176^{b}	2.954 ± 0.123^{b}
150	–	–	3.706 ± 0.124^{a}	–

[a–e]Values (Mean ± S.D., $n = 3$) with different superscripts in separate group are significantly different ($p < 0.05$).
[f]Scavenging potency = scavenging effect (%)/solid concentration in the reaction.
[g]Absorbance at UV_{420nm} after dilution with distiled water to 20 folds.
[h]No test for the condition.
[i]Scavenging potency and absorbance at UV_{420nm} of fresh kumquat are 11.46 ± 0.53^{e} (%/mg/mL) and 0.384 ± 0.050^{e}.

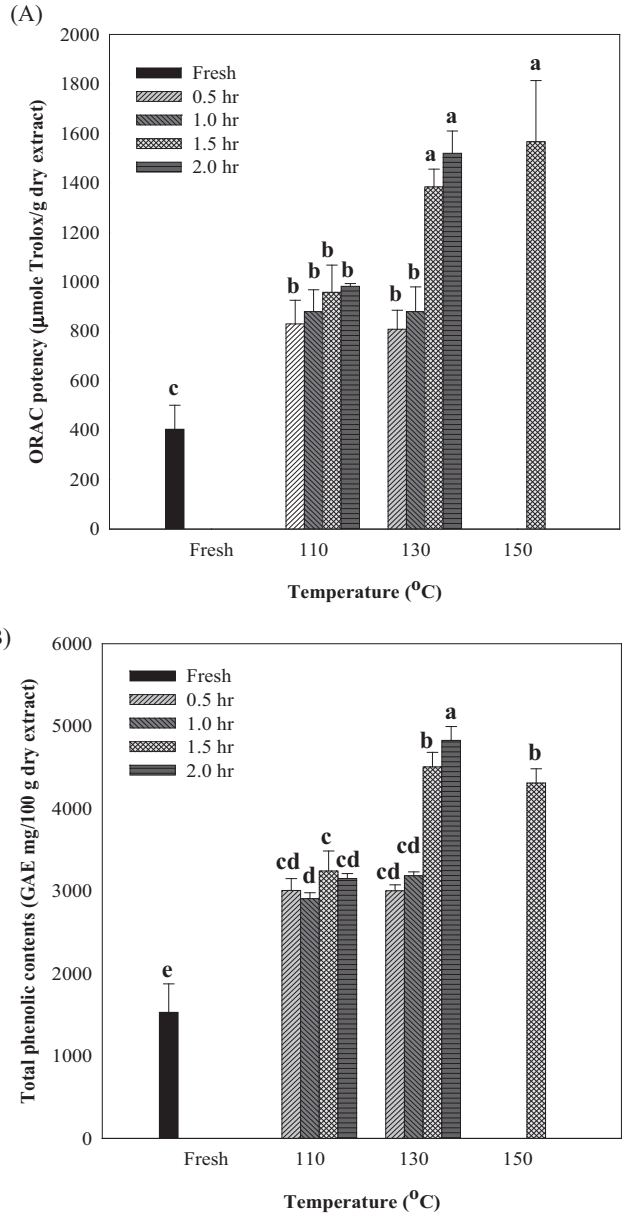

Fig. 2. (A) Oxygen radical absorbance capacity (ORAC) and (B) total phenolic content of hot water extracts from immature kumquat by different drying process. (a–e: different characters means significantly different ($p < 0.05$)).

flavonoid composition of hot water extracts from immature kumquat after drying at 110 °C are shown in Table 4. There were no obviously changes in the contents of DGPP and rhoifolin during drying up to 2.0 h. However, the contents of margaritene, isomargaritene, fortunellin, apigenin 8-*C*-neohesperidoside, and poncirin increased during first 0.5 h drying, i.e., 1768 ± 16 mg/100 g dry extract for margaritene to 2276 ± 307 mg/100 g dry extract, and 1547 ± 39 mg/100 g dry extract for isomargaritene to 2165 ± 304 mg/100 g dry extract. Then, there were no significantly changes when they were extended to 2 h drying. These data indicated that the flavonoids of immature kumquat could be better extracted after drying at 110 °C for a short time (i.e., 0.5 h), which might be due to the effect of the heat degradation to cellulose structure of the immature kumquat. No significant thermal degradation effect for the existed flavonoids was found during drying at 110 °C until 2.0 h, since most of the flavonoids of immature kumquat were *C*-glycoside derivatives, which were more heat resistant.

Table 4 also showed the changes in flavonoids of hot water extracts from immature kumquat after drying at 130 and 150 °C. For drying at 130 °C, the content of DGPP decreased remarkably after 1.5 h drying. The content decreased from 3792 ± 138 mg/100 g dry extract to 2032 ± 152 and 1708 ± 158 mg/100 g dry extract for drying 1.5 and 2.0 h, respectively. Similarly, the content of poncirin decreased slightly at 1.5 h drying. On the other hand, the contents of margaritene, isomargaritene, fortunellin, apigenin 8-*C*-neohesperidoside and poncirin increased during the first 1.0 h drying, then decreased significantly after 1.5 h drying. All of the flavonoids in immature kumquat decreased drastically after drying at 150 °C for 1.5 h. These revealed that the flavonoids in immature kumquat could be degraded by high temperature heating over 130 °C for more than 1.5 h. However, drying at 130 °C for 1.0 h, could enhance the yield of extraction for flavonoids, which also probably due to the destruction of the cell wall structure, i.e., cellulose and lignin. The content of flavonoids extracted at 130 °C for 1.0 h was higher than that at 110 °C for 0.5–2.0 h. Therefore, it is suggested that immature kumquat could be dried at 130 °C for 1.0 h before the hot water extraction process to obtain highest level of flavonoids with increased antioxidant activity.

3.5. Changes in total phenolic content and absorbance at UV_{420nm} during drying

The changes in total phenolic content of hot water extract from immature kumquat were shown in Fig. 2 (B). The total phenolic content of immature kumquat by drying at 110 °C was twice higher compared to the fresh immature kumquat. Interestingly, the total phenolic content of immature kumquat drying at 130 °C

Table 4
Changes of flavonoid composition in hot water extract of immature kumquat after drying at 110, 130 °C for different durations and 150 °C for 1.5 h.

Flavonoids	Drying time (hours)					at 150 °C
	0	0.5	1.0	1.5	2.0	1.5
	Contents (mg/100 g dry extract)					
Drying at 110 °C						
3′,5′-Di-*C*-β-glucopyranosylphloretin	3792 ± 138[a]	3753 ± 573[a]	3649 ± 426[a]	3500 ± 601[a]	3156 ± 160[a]	
Acacetin 8-*C*-neohesperidoside	1768 ± 16[b]	2276 ± 307[a]	2180 ± 70[a]	2294 ± 102[a]	2063 ± 38[ab]	
Acacetin 6-*C*-neohesperidoside	1,547 ± 39[b]	2165 ± 304[a]	2123 ± 88[a]	2181 ± 187[a]	1904 ± 93[a]	
Fortunellin	370 ± 12[b]	472 ± 56[a]	490 ± 23[a]	473 ± 62[a]	444 ± 29[ab]	
Apigenin 8-*C*-neohesperidoside	199 ± 34[b]	335 ± 46[a]	335 ± 7[a]	343 ± 29[a]	310 ± 30[a]	
Poncirin	66 ± 2[b]	94 ± 12[a]	93 ± 2[a]	91 ± 13[a]	81 ± 5[ab]	
Rhoifolin	29 ± 7[a]	31 ± 4[a]	30 ± 3[a]	31 ± 1[a]	29 ± 2[a]	
Total	8472 ± 171[b]	9768 ± 724[a]	9683 ± 442[a]	9887 ± 643[a]	9142 ± 200[a]	
Drying at 130 °C						
3′,5′-Di-*C*-β-glucopyranosylphloretin	3792 ± 138[a]	3860 ± 299[a]	3713 ± 85[a]	2032 ± 152[b]	1708 ± 158[b]	1006 ± 234[c]
Acacetin 8-*C*-neohesperidoside	1768 ± 16[bc]	2332 ± 90[a]	2359 ± 61[a]	2003 ± 237[ab]	1937 ± 265[ab]	1486 ± 399[c]
Acacetin 6-*C*-neohesperidoside	1547 ± 39[b]	2244 ± 44[a]	2241 ± 75[a]	1493 ± 174[b]	1399 ± 186[b]	739 ± 195[c]
Fortunellin	370 ± 12[b]	509 ± 16[a]	522 ± 19[a]	378 ± 43[b]	366 ± 47[b]	244 ± 63[c]
Apigenin 8-*C*-neohesperidoside	199 ± 34[b]	344 ± 10[a]	346 ± 10[a]	266 ± 63[ab]	256 ± 72[b]	181 ± 45[b]
Poncirin	66 ± 2[a]	72 ± 4[a]	72 ± 6[a]	42 ± 5[b]	41 ± 6[b]	27 ± 7[c]
Rhoifolin	29 ± 7[c]	33 ± 4[c]	33 ± 3[c]	58 ± 4[ab]	69 ± 8[a]	49 ± 14[b]
Total	8472 ± 171[c]	10,209 ± 317[a]	10,430 ± 134[a]	9500 ± 484[b]	8927 ± 494[bc]	5823 ± 598[d]

[a–c]Values (Mean ± S.D., $n = 3$) in the same row with different superscripts are significantly different ($p < 0.05$).

after 1.5 h increased significantly. Similar change was found at 150 °C for 1.5 h. This phenomenon coincided with the changes of DPPH scavenging potency and ORAC value. Thus, the correlation coefficient between total phenolic content and ORAC was significant with $r = 0.9720$ ($p < 0.001$), while it was 0.6197 between total phenolic content and DPPH scavenging potency.

It is therefore suggested that the high total phenolic content might contribute to the high antioxidant activity by high temperature drying. However, the flavonoid compounds decreased by high temperature drying, which lead to a reasonable interpretation that some unidentified compounds might also contribute to the antioxidant activity.

The colour of hot water extract from drying immature kumquat was remarkably darker than fresh kumquat. Therefore, the changes in absorbance at UV_{420nm} of hot water extract from immature kumquat after drying were investigated (Table 3). The absorbance increased after drying at 110 °C for 1.5 h. However, the absorbance also increased at 130 °C drying for 1.0 h, while a drastically increase of the absorbance was observed at 130 °C drying for more than 1.5 h, or drying at 150 °C for 1.5 h. Consequently, significant correlation coefficient (r) between the absorbance at UV_{420nm} and DPPH scavenging potency as well as ORAC was found, which were 0.8572 ($p < 0.01$) and 0.9164 ($p < 0.001$), respectively.

These hinted that products of browning reaction might also provide the antioxidant activity, since an increasing browning colour of the hot water extract at high temperature drying (over 130 °C for 1.5 h) was observed. It has also been found that the absorbance of UV_{420nm}, as browning index, increased significantly at 130 °C for 1.5 h in immature calamondin (Shie, 2012). Thus, the effect of browning products should also be considered and studied more detail in the future.

4. Conclusion

The major flavonoids of immature kumquat were DGPP, margaritene, and isomargaritene, which contained 48.2%, 22.9%, and 20.0%, respectively. Drying effect with temperature lower than 130 °C for 1.5 h, the antioxidant activity of hot water extract from immature kumquat could be enhanced, which might be due to the increase of phenolic compounds. The antioxidant activity of kumquat could be drastically increased, when the drying effect is more than 130 °C for 1.5 h. It is suggested that products of browning reaction might play an important role.

Acknowledgement

This research was supported by the Ministry of Science and Technology, Republic of China, under Grant No. MOST-103-2221-E-197-016.

References

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U., & Gattuso, G. (2010). Flavonoid composition and antioxidant activity of juices from Chinotto (*Citrus × myrtifolia* Raf.) fruits at different ripening stages. *Journal of Agricultural and Food Chemistry, 58*, 3031–3036.

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U., & Gattuso, G. (2011). Kumquat (*Fortunella japonica* Swingle) juice: Flavonoid distribution and antioxidant properties. *Food Research International, 44*, 2190–2197.

Chao, W. P. (2011). Antioxidant activities and active compounds in kumquat affected by drying processing. Master Thesis, Department of Food Science, National Ilan University, Taiwan.

Chiu, N. C., & Chang, K. S. (1998). *The illustrated medicinal plants of Taiwan*. Taiwan: SMC publishing Ltd.

Cho, J. Y., Kawazoe, K., Moon, J. H., Park, K. H., Murakami, K., & Takaishi, Y. (2005). Chemical constituents from the fruit peels of *Fortunella japonica*. *Food Science and Biotechnology, 14*, 599–603.

Choi, M. Y., Chai, C., Park, J. H., Lim, J., Lee, J., & Kwon, S. W. (2011). Effects of storage period and heat treatment on phenolic compound composition in dried citrus peels (Chenpi) and discrimination of Chenpi with different storage periods through targeted metabolomic study using HPLC-DAD analysis. *Journal of Pharmaceutical and Biomedical Analysis, 54*, 638–645.

Ferreres, F., Silva, B. M., Andrade, P. B., Seabra, R. M., & Ferreira, M. A. (2003). Approach to the study of C-glycosyl flavones by ion trap HPLC-PAD-ESI/MS/MS: Application to seeds of quince (*Cydonia oblonga*). *Phytochemical Analysis, 14*, 259–352.

Gil-Izquierdo, A., Gil, M. I., & Ferreres, F. (2002). Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. *Journal of Agricultural and Food Chemistry, 50*, 5107–5114.

Ho, S. C., & Lin, C. C. (2008). Investigation of heat treating conditions for enhancing the anti-inflammatory activity of citrus fruit (*Citrus reticulate*) peels. *Journal of Agricultural and Food Chemistry, 56*, 7976–7982.

Hsu, Y. L., Kuo, P. L., & Lin, C. C. (2004). Acacetin inhibits the proliferation of Hep G2 by blocking cell cycle progression and inducing apoptosis. *Biochemical Pharmacology, 67*, 823–829.

Jayaprakasa, K. G., Murthy, K. N. C., Etlinger, M., Mantur, S. M., & Patil, B. S. (2012). Radical scavenging capacities and inhibition of human prostate (LNCaP) cell proliferation by *Fortunella margarita*. *Food Chemistry, 131*, 184–191.

Jeong, S. M., Kim, S. Y., Kim, D. R., Jo, S. C., Nam, K. C., & Ahn, D. U. et al. (2004). Effect of heat treatment on the antioxidant activity of extracts from citrus peels. *Journal of Agricultural and Food Chemistry, 52*, 3389–3393.

Kawail, S., Tomono, Y., Katase, E., Ogawa, K., & Yano, M. (1999). Quantitation of flavonoid constituents in Citrus fruits. *Journal of Agricultural and Food Chemistry, 47*, 3565–3571.

Kim, J. S., & Lee, Y. S. (2009). Antioxidant activity of Maillard reaction products derived from aqueous glucose/glycine, diglycine, and triglycine model systems as a function of heating time. *Food Chemistry, 116*, 227–232.

Kumamoto, H., Matsubara, Y., Iizuka, Y., Okamoto, K., & Yokoi, K. (1985). Structure and hypotensive effect of flavonoids glycosides in kinkan (*Fortunella japonica*) peelings. *Agricultural and Biological Chemistry, 49*, 2613–2618.

Liao, Y. H., Houghton, P. J., & Hoult, J. R. (1999). Novel and known constituents from Buddleja species and their activity against leukocyte eicosanoid generation. *Journal of Natural Products, 62*, 1241–1245.

Lin, C. C., Hung, P. F., & Ho, S. C. (2008). Heat treatment enhances the NO-suppressing and peroxynitrite-intercepting activities of kumquat (*Fortunella margarita* Swingle) peel. *Food Chemistry, 109*, 95–103.

Lou, S. N., Lin, Y. S., Hsu, Y. S., Chiu, E. M., & Ho, C. T. (2014). Soluble and insoluble phenolic compounds and antioxidant activity of immature calamondin affected by solvents and heat treatment. *Food Chemistry, 161*, 246–253.

Lou, S. N., Yu, M. W., & Ho, C. T. (2012). Tyrosinase inhibitory components of immature calamondin peel. *Food Chemistry, 135*, 1091–1096.

Ma, Y. L., Cuyckens, F., Van den Heuvel, H., & Claeys, M. (2001). Mass spectrometric methods for the characterisation and differentiation of isomeric *O*-diglycosyl flavonoids. *Phytochemical Analysis, 12*, 159–165.

Ogawa, K., Kawasaki, A., Omura, M., & Yoshida, T. (2001). 3′,5′-Di-*C*-β-glucopyranosylphloretin, a flavonoid characteristic of the genus *Fortunella*. *Phytochemistry, 57*, 737–742.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*, 4619–4626.

Pan, M. H., Lai, C. S., Wang, Y. J., & Ho, C. T. (2006). Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice. *Biochemical Pharmacology, 72*, 1293–1303.

Ramful, D., Tarnus, E., Aruoma, O. I., Bourdon, E., & Bahorun, T. (2011). Polyphenol composition, vitamin C content and antioxidant capacity of Mauritian citrus fruit pulps. *Food Research International, 44*, 2088–2099.

Rezk, B. M., Haenen, G. R. M. M., Van der Vijgh, W. J. F., & Bast, A. (2002). The antioxidant activity of phloretin: The disclosure of a new antioxidant pharmacophore in flavonoids. *Biochemical and Biophysical Research Communications, 295*, 9–13.

Roowi, S., & Crozier, A. (2011). Flavonoids in tropical citrus species. *Journal of Agricultural and Food Chemistry, 59*, 12217–12225.

Sadek, E. S., Makris, D. P., & Kefalas, P. (2009). Polyphenolic composition and antioxidant characteristics of kumquat (*Fortunella margarita*) peel fractions. *Plant Foods for Human Nutrition, 64*, 297–302.

Shao, X., Bai, N., He, K., Ho, C. T., Yang, C. S., & Sang, S. (2008). Apple polyphenols, phloretin and phloridzin: New trapping agents of reactive dicarbonyl species. *Chemical Research in Toxicology, 21*, 2042–2050.

Shie, S. P. (2012). *Antioxidant activities and effective compounds in immature calamondin affected by drying* (Master thesis). Taiwan: Department of Food Science, National Ilan University.

Taga, M. S., Miller, E. E., & Pratt, D. E. (1984). Chia seeds as a source of natural lipid antioxidants. *Journal American Oil Chemistry Society, 61*, 928–931.

Truchado, P., Vit, P., Ferreres, F., & Tomas-Barberan, F. (2011). Liquid chromatography-tandem mass spectrometry analysis allows the simultaneous characterization of *C*-glycosyl and *O*-glycosyl flavonoids in stingless bee honeys. *Journal of Chromatography A, 1218*, 7601–7607.

Xu, G. H., Ye, X. G., Chen, J. C., & Liu, D. H. (2007). Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. *Journal of Agricultural and Food Chemistry, 55*, 330–335.

Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience Biotechnology Biochemistry, 62*, 12011204.

Yu, M. W., Lou, S. N., & Ho, C. T. (2013). Antioxidant activity and effective compounds of immature calamondin peel. *Food Chemistry, 136*, 1130–1135.

Zang, K. T. (2005). *Small encyclopedia of Chinese herbal medicine*. Heliopolis Culture Group, Taiwan: Neptune Culture Publishing Ltd..