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RESEARCH NOTE

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Analysis of Flavonoids in Concentrated Pomegranate Extracts by HPLC with Diode Array Detection

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Abstract Three flavonoid compounds – quercetin, luteolin, and kaempferol – were analyzed from two commercially available concentrated pomegranate extracts produced in Turkey and Italy, respectively. The samples were freeze-dried and hydrolyzed by 0.4 M hydrochloric acid in 50% ethanol at 80°C. HPLC (high-performance liquid chromatography) with DAD (diode array detection) at a wavelength of 260 nm was used for the detection of the three flavonoids. The detection limits of the three compounds were in hundreds of picograms and the signal-to-noise ratio ranged from 4 to 5: quercetin: >308 pg, s/n=4.0; luteolin: >262 pg, s/n=4.5; kaempferol: >688 pg, s/n=5.0. Quercetin, but not luteolin and kaempferol, was detected in the both pomegranate extracts. The concentrations of quercetin were 49.7 μg/g and 27.7 μg/g in the two pomegranate extracts made in Turkey and Italy, respectively.

Keywords: pomegranate, quercetin, flavonoid, HPLC

Introduction

Flavonoids are ubiquitous in the plant kingdom and have been identified in a large variety of fruits and vegetables. A number of studies report that flavonoids have several health benefits, including antioxidative (1-3), anticancer (4-5), and antiviral properties (6-7). Flavonoids are a major group of polyphenolic compounds with a basic diphenylpropane structure (C_6-C_3-C_6). Flavonoid contents have been presented constantly in diverse plants and food ingredients such as vegetables and fruits (8-11). Flavonoids are "phytoestrogens" because they are plant-derived compounds which show estrogen-like properties similar to endogenous mammalian estrogens. In particular, Rosenberg Zand *et al.* (12) reported significant steroidal hormone activity of flavonoids including flavones (e.g. luteolin), flavonols (e.g. quercetin), and isoflavones (e.g. genistein). Some have suggested that the estrogenicity of flavonoidic polyphenols is related to specific properties of the molecular structure (12) as well as structural similarity with the mammalian steroidal hormones (13-14).

The pomegranate (*Punica granatum*) belongs to the Punicaceae family and is often consumed unprocessed or as juices, jams, syrups, and liquors. Pomegranate consumption as a juice has increased among the general Korean population because of the reported health benefits. Antioxidative and antimutagenic properties of the pomegranate as well as adjuvant therapeutic potential for human breast cancer have been reported (15-17). Studies have attributed the reported disease preventing benefits of the pomegranate to diverse phenolic and polyphenolic compounds (e.g. flavonoids, tannins) (15-18). Gil *et al.* (15) and Poyrazoğlu *et al.* (19) reported that the contents of flavonoids in pomegranate juice. More recently, van Elswijk *et al.* (20) reported the presence of three phytoestrogenic flavonoids in pomegranate peel extracts (Fig. 1) – luteolin (129 μM), quercetin (140 μM), and kaempferol (230 μM). This study was designed to identify and quantify the three phytoestrogenic flavonoids - quercetin, luteolin, and kaempferol - in two commercial pomegranate extracts that are widely consumed in Korea as assayed by HPLC coupled with diode array detection.

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Materials and Methods

Chemicals Quercetin dihydrate, luteolin and BHT (butylated hydroxytoluene) were obtained from Sigma Chemical Co. (Steinheim, Germany) and kaempferol from Fluka (Steinheim, Germany). Hydrochloric acid and 96% ethyl alcohol were purchased from Riedel-de Haën (Seelze, Germany). Methanol and formic acid of HPLC grade was purchased from Mallinckrodt Inc. (Phillipsburg, NJ, USA) and Fluka (Switzerland), respectively. Water was purified via a Purelab Classic system (High Wycombe, Bucks, UK).

Preparation of standard solution The flavonoid standards were dissolved in methanol and stored at -20°C. The purity of the standards was determined by dividing the peak area of the reference compounds by all peak areas (standards, impurities, and byproducts) in the HPLC chromatogram. All compounds were over 97% pure. Calibration solutions were prepared before every period of analysis. The stock solution for calibration was diluted to 10 mL with 50% ethanol.

Sample preparation The two pomegranate extracts made in Italy (Philipp Tropenfrucht A. G. Co.) and Turkey (Goknur A. S. Co.) were obtained from food trading companies in Korea. Approximately two grams of the pomegranate extract was accurately weighed and lyophilized. Samples were prepared in triplicate. All extracts were prepared as follows: 5 mL of 0.4 M HCl in 50% EtOH (containing 0.05% BHT) was added to the freeze-dried samples, then

50 μL of 96% ethanol containing *n*-butyrophenone (internal standard, 0.01 g/10 mL) was added to each sample. The mixtures were hydrolyzed by heating at 80°C for 0.5, 1, 1.5, 2, and 2.5 hr on a water bath, which was allowed to rapidly cool under cold running tap water. After cooling, the samples were blended by a vortex mixer for 30 s to form the final extract. Approximately 0.4 mL was filtered through a 0.45-μm PVDF filter unit (Whatman Inc. Clifton, New Jersey, USA) and a 20-μL portion of these solutions was injected into the HPLC system.

HPLC Samples were analyzed using an Agilent instrument (Waldbronn, Germany) with a 1100 series quaternary pump, an autosampler and a diode array detector linked to an Agilent ChemStation data handling system. Reversed-phase separations were carried out using an ODS Hypersil column (200 × 4.6 mm i.d., 5 μm, Thermo Electron Corporation, Bellefonte, PA, USA) fitted with a guard column (ODS-Hypersil, 20 × 4.0 mm i.d., 5 μm, Agilent, USA). The column was placed in a column compartment set at 25°C. Two solvents, A (1% formic acid) and B (methanol), were used in a linear gradient of 40%~86% B (65 min) at a flow rate of 1 mL/min. The flavonoids were detected at 260 nm and identified according to the retention times and UV spectra of the standards. Spectra were recorded upslope, apex, and downslope (240-450, 2 nm steps). Quantification was performed using the calibration curves obtained from the ratio of the UV responses of the internal standard and the identified compound.

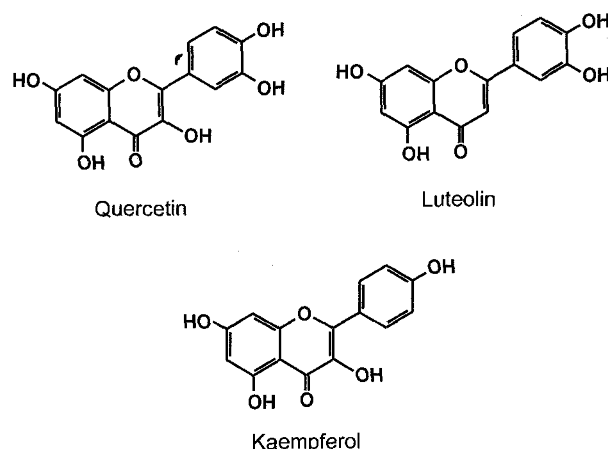

Fig. 1. Chemical structures of the flavonoids quercetin, luteolin, and kaempferol.

Results and Discussion

Identification of quercetin, luteolin, and kaempferol Table 1 shows retention times, capacity factors, relative retentions, plate numbers, resolutions, UV maxima, and detection limits of the three major flavonoid aglycones. Some researchers have tried increasing running times and/or changing the constitution of the eluents for satisfactory peak separation between quercetin and luteolin on the reversed-phase HPLC because the two peaks overlap in certain conditions (21-24) due to similar structural similarity (Fig. 1). The two peaks were not fully separated in the study by Crozier *et al.* (21) and Swatsitang *et al.* (23). Kim *et al.* (24) separated the two peaks adjacently (quercetin: t_R 33.96 min; luteolin: t_R 35.51 min) after over 30 min of retention. However, we obtained good separate peaks of the two flavonoids in 20 min (Table 1, Fig. 2. A). More recently, Wang *et al.* (26) reported the separation results of nine flavonoids including quercetin and luteolin by HPLC using THF as an additive in aqueous mobile phase. In their study, the two peaks of flavonoids (quercetin and luteolin) were separated in 10 min, but the relative retention between the two peaks was lower than our study. The conditions set in our study (see the **HPLC** section) is effective in analyzing samples that contain both quercetin and luteolin. Detection limits of the three flavonoids were measured in picograms (Table 1). The detection limit of quercetin in our study is lower than that of Sladkovský *et al.* (25).

The retention times of the peaks of flavonoids from the extracts were compared with those of the standard reference compounds. Identification of the peaks was confirmed by superimposing the UV spectrum of each peak with the corresponding standard spectrum (Fig. 2). Only quercetin was detected in the two samples. The spectra (b) and (c) matched well with the standard quercetin spectrum (a) in the absorption maxima of 258 and 370 nm. However, in the case of luteolin (Fig. 2e), the absorption pattern was completely different from the luteolin standard (Fig. 2d). Therefore, we concluded that the peak (e) is not of luteolin but one of unknown impurity. The peak of kaempferol did not appear in either sample.

Quantification of quercetin To determine optimum hydrolysis conditions, we varied the time for hydrolysis reaction - 0.5, 1, 1.5, 2, and 2.5 hr. The effective hydrolysis time was 1.5 hr in this experiment. The calibration curve of the quercetin standard ranging from 1.2 to 46 μg/mL (4

Table 1. Retention times (t_R), capacity factors (k'), relative retentions (α), plate numbers, resolutions (R), UV absorbance maxima (λ_{max}), and detection limits of the three flavonoids

Compounds	t_R (min)	k'	α	plate no.	R	λ_{max} (nm)	Detection limits			
							ng/mL	pg	ng/mL	pg
Quercetin	15.9	4.48		14866		258, 370	15.4[a]	308[a]	45[b]	450[b]
Luteolin	18.3	5.31	1.18	24166	4.99	255, 350	13.1[a]	262[a]	-[c]	-[c]
Kaempferol	22.3	6.69	1.25	19018	6.0	267, 368	34.4[a]	688[a]	50[b]	500[b]

[a]Determined with a 20 μL HPLC injection at a signal-to-noise ratio of 4.0 for quercetin, 4.5 for luteolin, and 5.0 for kaempferol (present study).
[b]Determined with a 10 μL HPLC injection based on a signal-to-noise ratio of 3 (Sladkovský *et al.*).
[a,b]Wavelength was set at 260 nm.
[c]Not reported by Sladkovský *et al.*

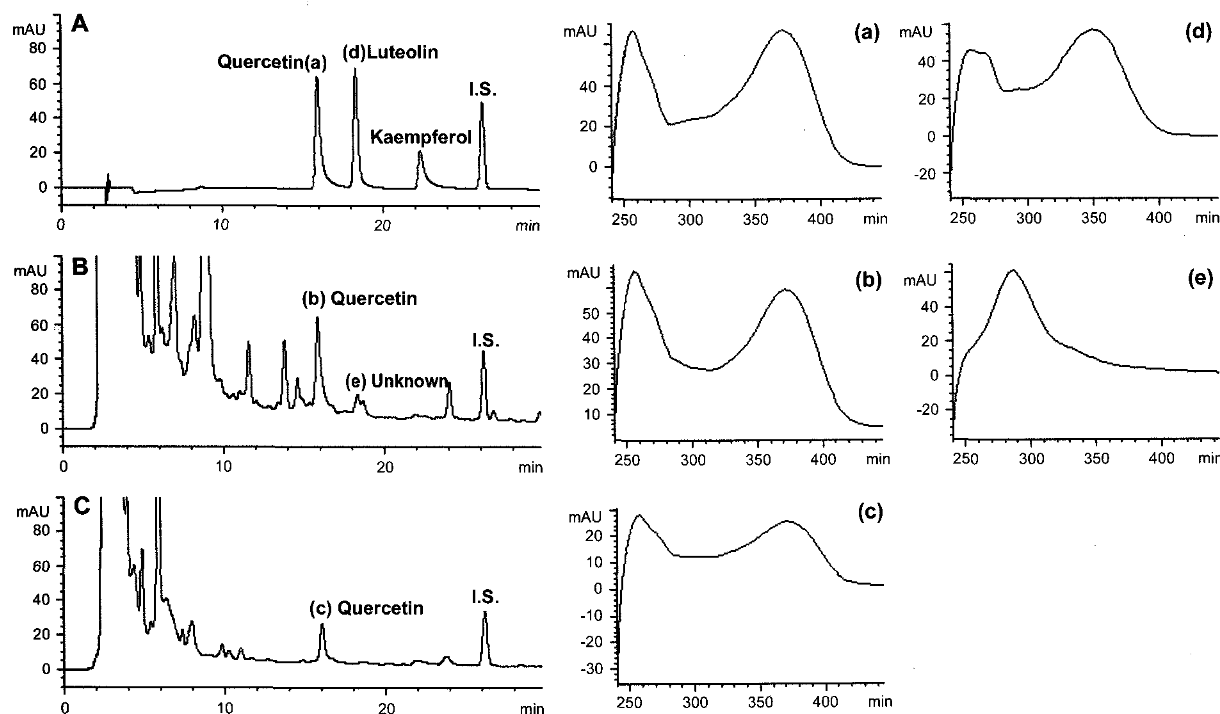

Fig. 2. Typical chromatogram of flavonoids (left) and their UV spectra (right). A: reference, B: hydrolysate of pomegranate extract from Turkey, C: hydrolysate of pomegranate extract from Italy. I.S.: *n*-butyrophenone internal standard.

levels) revealed a strong linear relationship with an r^2 value exceeding 0.997 (peak areas vs. concentration). The quantity of quercetin detected after an optimum hydrolysis period of 1.5 hr for both extracts was 49.7±1.1 μg/g (mean ±S.D., n=3, fresh weight) and 27.7±2.4 μg/g (mean±S.D., n=3, fresh weight) in the pomegranate extracts from Turkey and Italy, respectively. The difference in quercetin quantity in both extracts may be attributable to variations in manufacturing processes and to differences in the pomegranate related to the variety of fruit and cultivation characteristics. Quercetin recovery, determined in triplicate, was measured in the control samples by spiking pure standard to the extraction solutions prior to sample analysis. We obtained acceptable recoveries of 85.6±3.5% (mean±S.D., n=3) of the quercetin standard from the pomegranate extract made in Italy during the optimum hydrolysis period.

In conclusion, we identified and quantified the phyto-estrogenic flavonoids - quercetin, luteolin, and kaempferol - from two commercial pomegranate extracts by HPLC with diode array detection. The lower detection limit of the three flavonoids was in hundreds of picograms. Only the flavonol quercetin was found in the two kinds of samples. Quercetin was identified by comparison of UV patterns of the peaks with a known standard. Although quercetin, luteolin, and kaempferol are well described in literature, there have not been many reports on the flavonoid contents of pomegranates. The presence of quercetin in pomegranates may be partially responsible for the health benefits associated with pomegranate consumption. This paper provides information for the analysis of flavonoids in pomegranate samples.

References

1. Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med. 9: 441-449 (1990)
2. Ryu BH. Antioxidative activity of flavonoid on oxidation of human low density lipoprotein induced by macrophages and copper. Food Sci. Biotechnol. 11: 84-88 (2002)
3. Lee SM, Kim SS, Oh MS, Kim ES, Koh KH. Antioxidant effect of quercetin with rice bran oil on high cholesterol-fed rats. Food Sci. Biotechnol. 11: 490-493 (2002)
4. Yeh YA, Herenyiova M, Weber G. Quercetin: Synergistic action with carboxyamidotriazole in human breast carcinoma cells. Life Sci. 57: 1285-1292 (1995)
5. Singhal RL, Yeh A, Prajda N, Olah E, Sledge GW Jr., Weber G. Quercetin down-regulates signal transduction in human breast carcinoma cells. Biochem. Biophys. Res. Commun. 208: 425-431 (1995)
6. Kaul TN, Middleton E Jr., Ogra PL. Antiviral effect of flavonoids on human viruses. J. Med. Virol. 15: 71-79 (1985)
7. Wu JH, Wang XH, Yi YH, Lee KH. Anti-AIDS agents 54. A potent anti-HIV chalcone and flavonoids from genus *Desmos*. Bioorg. Med. Chem. 13: 1813-1815 (2003)
8. Bilyk A, Sapers GM. Distribution of quercetin and kaempferol in lettuce, kale, chive, garlic chive, leek, horseradish, red radish, and cabbage tissues. J. Agric. Food. Chem. 33: 226-228 (1985)
9. Chu YA, Chang CL, Hsu HF. Flavonoid content of several vegetables and their antioxidant activity. J. Sci. Food. Agric. 80: 561-566 (2000)
10. Trichopoulou A, Vasilopoulou E, Hollman P, Chamalides Ch, Foufa E, Kaloudis Tr, Kromhout D, Miskaki Ph, Petrochilou I, Poulima E, Stafilakis K, Theophilou D. Nutritional composition and flavonoid content of edible wild greens and green pies: a potential rich source of antioxidant nutrients in the Mediterranean diet. Food Chem. 70: 319-323 (2000)
11. Lee JH, Lee JY, Kim KN, Kim HS. Quantitative analysis of two major flavonoid aglycones in acid hydrolyzed samples of *Angelica keiskei* by HPLC. Food Sci. Biotechnol. 12: 415-418 (2003)
12. Rosenberg Zand RS, Jenkins DJA, Diamandis EP. Steroid hormone activity of flavonoids and related compounds. Breast

Cancer Res. Treat. 62: 35-49 (2000)
13. Messina MJ, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. Nutr. Cancer. 21: 113-129 (1994)
14. Shutt DA, Cox RI. Steroid and phyto-oestrogen binding to sheep uterine receptors in vitro. J. Endocrinol. 52: 299-310 (1972)
15. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J. Agric. Food Chem. 48: 4581-4589 (2000)
16. Negi PS, Jayaprakasha GK, Jena BS. Antioxidant and antimutagenic activities of pomegranate peel extracts. Food Chem. 80: 393-397 (2003)
17. Kim ND, Mehta R, Yu W, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, Mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E. Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast Cancer Res. Treat. 71: 203-217 (2002)
18. Schubert SY, Lansky EP, Neeman I. Antioxdant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. J. Ethnopharmacol. 66:11-17 (1999)
19. Poyrazoğlu E, Gökmen V, Artėk N. Organic acids and phenolic compounds in pomegranates (*Punica granatum* L.) grown in Turkey. J Food Comp. Anal. 15: 567-575 (2002)
20. van Elswijk DA, Schobel UP, Lansky EP, Irth H, van der Greef J. Rapid dereplication of estrogenic compounds in pomegranate (*Punica granatum*) using on-line biochemical detection coupled to mass spectrometry. Phytochemistry 65: 233-241 (2004)
21. Crozier A, Jensen Einar, Lean MEJ, McDonald MS. Quantitative analysis of flavonoids by reversed-phase high-perfromance liquid chromatography. J. Chromatogr. A. 761: 315-321 (1997)
22. Hertog MG, Hollman PCH, Venema DP. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. J. Agric. Food Chem. 40: 1591-1598 (1992)
23. Swatsitang P, Tucker G, Robards K, Jardine D. Isolation and identification of phenolic compounds in *Citrus sinensis*. Anal. Chim. Act. 417: 231-240 (2000)
24. Kim HY, Kang MH. Flavonoid content of some Korean medicinal plants. Food Sci. Biotechnol. 12: 687-690 (2003)
25. Sladkovský R, Solich P, Opletal L. Simultaneous determination of quercetin, kaempferol and (*E*)-cinnamic acid in vegetative organs of *Schisandra chinensis* Baill. by HPLC. J. Pharm. Biomedic. Anal. 24: 1049-1054 (2001)
26. Wang SP, Huang KJ. Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis. J. Chromatogr. A. 1032: 273-279 (2004)