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Determination of hesperidin in mixed tea by HPLC

Saem Han[1], So-Youn Mok[1], Hye-Min Kim[1], Jeong-Min Lee[1], Dong Gu Lee[1], Sook-Young Lee[2], Jongkee Kim[1], Sun-Ju Kim[3], Sanghyun Lee[1]*

[1]Department of Integrative Plant Science, Chung-Ang University, Anseong 456-756, Korea
[2]Department of Food and Nutrition, Chung-Ang University, Anseong 456-756, Korea
[3]Department of Bio-Environmental Chemistry, Chungnam National University, Daejeon 305-764, Korea

HPLC를 이용한 혼합차의 Hesperidin 정량

한 샘[1] · 목소연[1] · 김혜민[1] · 이정민[1] · 이동구[1] · 이숙영[2] · 김종기[1] · 김선주[3] · 이상현[1]*

[1]중앙대학교 식물시스템과학과, [2]중앙대학교 식품영양학과, [3]충남대학교 생물환경화학과

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Abstract : The content of hesperidin in the mixed tea, which was composed of dried orange peel, laurel leaf, mulberry leaf, silver magnolia leaf, oriental melon tap, cassia seed, and licorice root, was determined by high performance liquid chromatography (HPLC). Hesperidin was quantified by a reverse phase column with gradient solvent system (water:acetonitrile = 80:20 to 35:65 for 30 min) and UV/VIS detection (280 nm). The flow rate was kept constant at 1.0 ml/min. The content of hesperidin in the mixed tea was measured in depending on extraction time 1, 2, 3, and 4 min (29.07, 52.39, 52.45, and 88.35 mg/g, respectively).

Key words **:** Hesperidin, Mixed tea, Quantitative analysis, HPLC

I. Introduction

Tea is the most popular beverage, like coffee or soft drinks, consumed by over two thirds of the World's population. About three billion kilograms of tea is produced and consumed yearly (Naghma and Hasan, 2007). Recently, a large amount of information on tea has been published concerning the effects of tea and its major constituents on human health. This beverage has been consumed in many countries for a very long time and today, interest is growing because scientific reports indicate that tea could bring benefits for human health (Hollman et al., 1996). The aging of the population and limitations of modern medicine have caused many people to look for new ways to improve their health and life. Doubts surrounding lifestyle and diet along with the growing interest in functional foods and nutraceuticals have contributed to these trends (Dufresen and Farnworth, 2000). When we study the role of foods and folk medicines, we often discover that many foods and beverages were used for their assumed beneficial effects on health and life. Tea is the oldest known medicine in human life. It was taken in China 5000 years ago for its stimulating and detoxifying properties in the elimination of alcohol and toxins, to relieve joint pains, and to improve blood and urine flow (Balentine et al., 1997).

Recently, we make new mixed tea with different plant materials, such as orange peel, laurel leaf, mulberry leaf, silver magnolia leaf, oriental melon tap, cassia seed, and licorice root. In a previous

***Corresponding author:** Tel: +82-31-670-4688
E-mail address: slee@cau.ac.kr

report, this mixed tea was effective in evacuation of human, and has antioxidant activity (Kim et al., 2010). Among the plant materials in the mixed tea, orange peel was contained as a major material. The main biological active compound in orange peel is hesperidin. Hesperidin is flavonoid glycoside, which is composed of a flavonoid skeleton and two different sugar moiety. It is clear that hesperidin plays a role in reducing the cellular triacylglycerol and cholesterol contents in human hepatocyte because cholesterol acyltransferase, which is the enzyme for lipid metabolism, was blocked (Kim et al., 1999). Therefore, hesperidin, a main compound of orange peel, was selected as a marker compound in mixed tea.

Our aim of this study was the determination of hesperidin in mixed tea by HPLC.

II. Materials and methods

1. Apparatus and reagents

EI-MS spectra were conducted on a JEOL JMS-600W mass spectrometer (Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany). Chemical shifts are shown as δ-values (ppm) with TMS (tetramethylsilane) as an internal standard, and coupling constants (*J*) were expressed in hertz. TLC was performed with precoated silica gel 60 F_{254} plates (Art. 5715, Merck Co., Darmstadt, Germany) for the analysis. The compounds on TLC plate were visualized by spraying with 10% sulfuric acid in methanol followed by heating at 100℃ to detect spot color. Silica gel (200-400 mesh ASTM; Merck Co., Darmstadt, Germany) was used for open column chromatography. HPLC data were recorded on a Waters 1525 Binary HPLC Pump (Miami, USA) equipped with a Waters 2489 UV/VIS detector (Miami, USA). Water and acetonitrile used in this research were of HPLC grade, and all other reagents, such as *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and methanol, were of analytical grade.

2. Isolation of hesperidin

The sliced and dried peels of *C. unshiu* (3096.7 g) were extracted with methanol (8 L × 7) under reflux. After filteration and removal of solvent *in vacuo*, the methanol extracts were collected. The methanol extracts (727.4 g) were suspended in distilled water and then partitioned in turn using *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. A portion of the ethyl acetate fraction (8.6 g) was chromatographed on silica gel column (No. 7734, 6 × 80 cm) using gradient solvent system with chloroform-methanol solvent system to afford 150 sub-fractions. The sub-fraction 94 (chloroform:methanol = 90:10) led to the isolation of hesperidin. Hesperidin was obtained by recrystallization with methanol.

Hesperidin: White powder. $C_{28}H_{34}O_{15}$; EI-MS (rel. int., %): *m/z* 610 $[M]^{+}$; ^{1}H-NMR (500 MHz, DMSO): 12.02 (1H, s, 5-OH), 9.10 (1H, s, 3'-OH), 6.93 (3H, m, H-2',5',6'), 6.14 (1H, d, *J* = 2.1 Hz, H-6), 6.12 (1H, d, *J* = 2.1 Hz, H-8), 5.50 (1H, dd, *J* = 12.3, 3.0 Hz, H-2), 4.52 (1H, s, rhm-1), 3.77 (3H, s, 4'-OMe), 3.25 (1H, m, H-3b), 2.76 (1H, dd, *J* = 17.1, 5.5 Hz, H-3a), 1.08 (3H, d, *J* = 10.0 Hz, rhm-6); ^{13}C-NMR (125 MHz, DMSO): 197.0 (C-4), 165.1 (C-7), 163.0 (C-5), 162.5 (C-9), 147.9 (C-4'), 146.4 (C-3'), 130.9 (C-1'), 117.9 (C-6'), 114.1 (C-2'), 112.1 (C-5'), 103.3 (C-10), 100.6 (rhm-1), 99.4 (glc-1), 96.4 (C-6), 95.6 (C-8), 78.4 (C-2), 76.3 (glc-3), 75.5 (glc-5), 73.0 (glc-2), 72.1 (rhm-4), 70.7 (glc-4), 70.3 (rhm-2), 69.6 (rhm-3), 68.3 (rhm-5), 66.0 (glc-6), 55.7 (OMe), 42.2 (C-3), 17.9 (rhm-6).

3. Preparation of tea bag

A tea bag was a mixed tea with different plant materials. The mixed tea (1.2 g) was composed of dried orange peel (0.4 g, 33.3%), laurel leaf (0.3 g, 25.0%),

mulberry leaf (0.15 g, 12.5%), silver magnolia leaf (0.1 g, 8.3%), oriental melon tap (0.1 g, 8.3%), cassia seed (0.1 g, 8.3%), and licorice root (0.05 g, 4.2%).

4. Sample preparation

To quantify the amounts of hesperidin in mixed tea extracts depending on different extraction time, each tea bag was extracted with water (100℃) from 1 min to 4 min. After removal of water *in vacuo*, four crude extracts of different extraction time were yielded. The resultant solutions were filtered through a Whatman 0.45 μm PVDF syringe filter (Catalog No. 6779) prior to HPLC.

5. Quantitative analysis of hesperidin

The HPLC separation of hesperidin in the mixed tea extracts depending on extraction time for qualitative and quantitative analysis was performed using a reverse phase system. A reverse phase column (Nucleodur C_{18}, 5 μm, 250 × 4.6 mm, Macherey-nagel) was used, and a gradient solvent system (water: acetonitrile, v/v) was employed as the mobile phase. The gradient solvent system was 80:20 initially and was increased in linear gradients to 35:65 for 30 min. The flow rate was kept constant at 1.0 ml/min., and the peaks were identified using UV absorbance at 280 nm. The injection volume was 20 μl of prepared methanol solutions. HPLC analyses were performed in triplicate.

6. Calibration

Standard stock solutions (3.3 mg/ml) of hesperidin was prepared in 33% aqueous methanol and repeatedly blended with the same solvent. The hesperidin levels were ascertained by comparing the integrated peak areas of the individual compound with those of a standard curve prepared from the corresponding standards. The peak area (Y), concentration (X, mg/ml), and mean values (n = 5) of the calibration functions of hesperidin was calculated.

III. Results and discussion

A column chromatography of the methanol extracts from the peels of *C. unshiu* led to isolation of hesperidin (Fig. 1). The presence of hesperidin was identified by mass, ^{1}H-, and ^{13}C-NMR spectroscopy. The molecular formula of $C_{28}H_{34}O_{15}$ was expected by a molecular ion peak at m/z 610. In the ^{1}H-NMR spectrum, the signal at δ 12.02 showed the 5-OH of the flavonoid A-ring. And two doublets at δ 6.14 and 6.12 indicated meta-coupling constant in A-ring by J value (2.1 Hz). However, ABX type signals (H-2', -5', and -6') in B-ring were overlapped at δ 6.93. Singlet of methoxy group showed at δ 3.77. The proton signals at δ 3.00 – δ 5.00 exhibit glycoside moiety. In the ^{13}C-NMR spectrum, the signal at δ 197.0 showed the C=O and δ 55.7 showed the methoxy group. The kind of glycoside moiety found rutinose by special carbon signal δ 103.3 (glucose) and δ 17.9 (rhamnose). Accordingly, this structure was elucidated as hesperidin by comparing its spectral data in the literature (Han et al., 2001).

Hesperidin was a major constituent in *Citrus* species (Eun et al., 1996; Elisa et al., 2007; Horie et al., 1986). The content of hesperidin in the the the tea extracts was determined by HPLC. The calibration curve for hesperidin is Y = 31654.6105X – 225.8944 (r^2 = 0.9995) (Fig. 2). Hesperidin was shown at the retention time 10.66 min in the mixhe tea extract chromatograms (Fig. 3). Table 1 shows that hesperidin

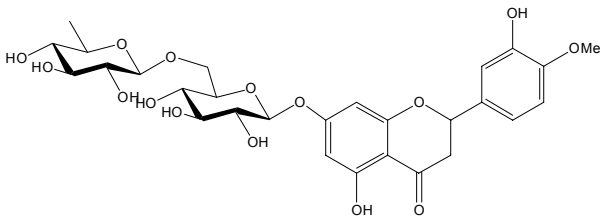

Fig. 1. Chemical structure of hesperidin.

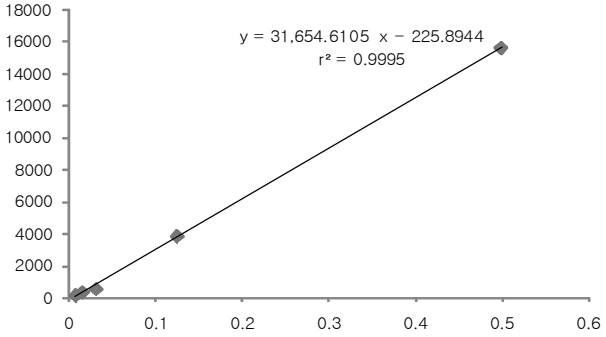

Fig. 2. Calibration curve for hesperidin (X axis, mg/20 μl; Y axis, Area).

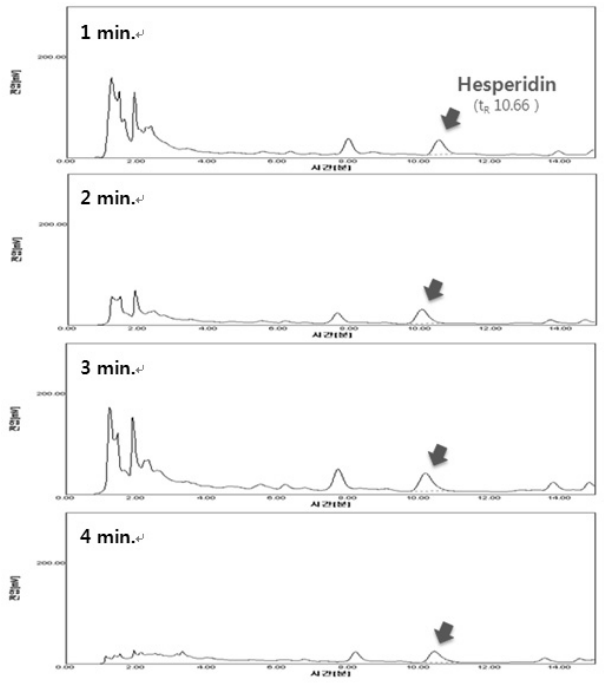

Fig. 3. HPLC chromatograms of the mixed tea extract by different extraction time.

was detected in the mixd tea extract of depending on different extraction time, 1 (29.07 mg/g), 2 (52.39 mg/g), 3 (52.45 mg/g) and 4 min (88.35 mg/g). From these results, it is assumed that the mixhe tea showed increasing contents of hesperidin by increasing extraction time.

The capacity of development and interaction between palatability and health effect of mixed tea were studied from 1980's. However, many new mixed teas were developed since 2000. Actually, mixed extract utilizing *Lycium chinense* and *Cornus officinalis* showed that the yeast growth and protective effect of liver

Table 1. Content of hesperidin in the mixed tea by different extraction time.

Extraction Time (min)	Hesperidin (mg/g)
1	29.07 ± 1.78
2	52.39 ± 3.88
3	52.45 ± 3.74
4	88.35 ± 6.88

Data are given as the mean ± S.D. (n = 5) in mg/g of mixed tea.

damage by mixed extract are proved to be better than single extract (Joo, 1988). In another case, mixed herb tea composed of *Chrysnathemum morifolium*, *Cornus officinalis*, and *Schizandra chinensis* showed that the increased transaminase release of CCl_4 treated hepatocytes was significantly lowered by mixed tea extract (Lee et al., 2011). From these results, the mixed tea has synergetic effect other than single tea.

Hesperidin, which has hypotensive effect (Son et al., 1992), antioxidant activities (Sohn and Kim, 2008), antiinflammatory activities (Monforte et al., 1995), and cholesterol reduction effect (Cha et al., 1999), has been identified in the mixed tea. The presence of hesperidin in the mixed tea extract is very important in beverage market for increasing the amounts of clinically available health supplements. Accordingly, these results prove that the mixed tea containing hesperidin has potential of development and commercializing of new drink as health supplement.

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