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Determination of Phenolic acids and Flavonol Aglycone Contents in *Orostachys japonicus* A. Berger Grown under Various Cultivation Conditions

Sang Hun Jang*, Sang Gyeong Lee*, Jin Ho Kang, Jong Cheol Park***, and Sung Chul Shin*†**

**Department of Chemistry and Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, South Korea.*
***Department of Agriculture and Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, South Korea.*
****Department of Oriental Medicine Resorces, Suncheon National University, Suncheon 540-741, South Korea.*

ABSTRACT : The content of four phonolic acids 1-4, and two flavonol aglycones 14 and 15 from *Orostachys japonicus* A. Berger grown under night-break and day-length controlled experiments was estimated and compared with those in wild plants. The amount of the phenolic acids 1-4 and the flavonol aglycones 14 and 15 increased with increasing light irradiation under both the night-break and day-length control conditions. It was disclosed that the cultivation conditions such as the night-break and the day-length control were not adversely affect the production of phenolic acids and flavonols in *Orostachys japonicus* A. Berger extracts.

Key words **:** *Orostachys japonicus* A. Berger, phenolic acids, flavonols, flavonol aglycones, HPLC

INTRODUCTION

Plant polyphenols are secondary metabolites that have aromatic rings bearing one or more hydroxy groups. Polyphenols range from simple molecules, such as phenolic acids, to highly polymerized compounds such as tannins. They are mainly present in a conjugated form, with one or more sugar residues linked to a hydroxy group. Unconjugated polyphenols are also found in many plants. They are of interest on account of their apparent health promoting effects including activities such as antioxidant (Frank *et al.*, 1993, Rice-Eva *et al.*, 1996), cancer inhibitor (Harbor *et al.*, 2000), vasodilator (Cheng *et al.*, 1993) and platelet disaggregator (Gryglews *et al.*, 1987). Epidemiological studies have reported a correlation between the increased consumption of polyphenolic antioxidants and the reduced risk of cardiovascular disease (Hertog *et al.*, 1993a, Hertog *et al.*, 1995, Hertog *et al.*, 1997) as well as certain types of cancers (Hertog *et al.*, 1994, Hertog *et al.*, 1995). One of the herbs frequently used in folk cancer remedies and health food in Korea is the *Orostachys japonicus* A. Berger (Kang *et al.*, 1995), where patients drink hot water extracts of the plant. Recently, the consumption of the *Orostachys japonicus* A. Berger has been increased greatly due to the increase in the number of cancer patients in Korea. Therefore, cultivating the *Orostachys japonicus* A. Berger is attractive to farmers as an important income producing crop. However, since the cultured *Orostachys japonicus A. Berger* and the wild one are released into the market at the same time (i.e. early October), the market price plummet. In order to prevent a sharp drop in the price, the time for releasing the plant to market needs to be controlled (Kang *et al.*, 1995, Kang *et al.*, 1996, Kang *et al.*, 1997).

Recently, Kang *et al* examined the retardation of *Orostachys japonicus* A. Berger growth through a night-break and a day-length adjustment, and temperature control during the winter season (Kang *et al.*, 1995, Kang *et al.*, 1996, Kang *et al.*, 1997). They showed that regulating such cultivation conditions retards the growth of the *Orostachys japonicus* A. Berger (Kang *et al.*, 1995, Kang *et al.*, 1996, Kang *et al.*, 1997). This means that the cultured *Orostachys japonicus* A. Bergers can be released into market at the farmer's discretion.

However, it is important to retain the quality of cultured *Orostachys japonicus* A. Bergers. Thus, the content of the health promoting components such as polyphenols should not be lower than in the wild one. In order to disclose their quality, this study investigated the content of the components of cultured *Orostachys japonicus* A. Bergers and compared it with the wild type plants.

The content of polyphenols-phenolic acids and flavonol aglycones in the *Orostachys japonicus* A. Berger samples col-

† Corresponding author: (Phone) +82-55-751-6022 (E-mail) scshin@nongae.gsnu.ac.kr
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lected from day-length control and night-break experiments were investigated using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Materials

The wild *Orostachys japonicus* A. Bergers were purchased at the Chinju central market on October 1, 2004. The *Orostachys japonicus* A. Bergers were transplanted into a greenhouse in the experimental farm at Gyeongsang National University on May 31, 2004. Each seedling was assigned to adifferent plastic pot of 18 cm diameter, which was filled with a soil and fertilizer blend (2 : 1, v/v). Sufficient water was supplied to the *Orostachys japonicus* A. Bergers every two or three days, and the ambient temperature was retained throughout the experiments. The standards for the phenolic acids, 4-hydroxybenzoic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), gallic acid (**3**) and methyl gallate (**4**), and flavonol aglycones, kaempferol (**14**) and quercetin (**15**), were purchased from Sigma-Aldrich Korea (Yongin, South Korea) and all the solvents were of HPLC grade.

Methods

Day-length control experiment

The *Orostachys japonicus* A. Bergers were divided to three sample groups according to the following day-length: 10/14 h, 13/11 h and 16/8 h (day/night) employing a light-curtain. The day-lengh experiment was carried out on each sample group from on August 25. An incandescent electric lamp with a luminosity 45 ㏖ $m^{-2}s^{-1}$ was used as the light source. The day-lengths, 10, 13 and 16 h, were from 8 a.m. to 6 p.m., from 6:30 a.m. to 7:30 p.m. and from 5 a.m. to 9 p.m., respectively.

The *Orostachys japonicus* A. Berger samples were gathered immediately after bursting into bloom. Thus, samples of the 10/14, 13/11 and 16/8 plants were collected on September 22, on October 22 and on November 4, respectively.

Night-break experiment

The *Orostachys japonicus* A. Bergers were divided to three sample groups, and the night-break experiment was carried out on each sample group from on June 30, July 28 and August 25. The sample was irradiated with 45 ㏖ $m^{-2}s^{-1}$ of light for two hours from 11 p.m. to 1 a.m. using an incandescent electric lamp. The *Orostachys japonicus* A. Bergers samples were gathered immediately after bursting into bloom. Accordingly, the samples planted at August 25 were collected on October 6 and those planted at June 30 and July 28 were collected on September 4.

Extraction and hydrolysis

The phenolic acids in the *Orostachys japonicus* A. Bergers were extracted by a modification of a method described in the literature (Price *et al.*, 1998). Briefly, 4 g of the dried powder of the *Orostachys japonicus* A. Bergers was homogenized three times in 70% methanol (100 mL) at 5000 rpm for 2 min (AM-7 homogenizer, Nihonseiki Kaisha Ltd, Japan) and the homogenate was filtered under reduced pressure through a filter paper (Whatman No1). The filtrate was concentrated under reduced pressure at 30 ℃ to approximately 15 mL, and the sample volume was increased to 20 mL with methanol.

The flavonol glycosides in the *Orostachys japonicus* A. Bergers were hydrolyzed by a modification of a method described in the literature (Hertog *et al.*, 1993b). Thus, 1.0 g of the *Orostachys japonicus* A. Bergers was mixed with 40 mL of 70% aqueous methanol and 5 mL 6 *M* HCl. After refluxing for 6 h, the solution was filtered into a 50 mL volumetric flask and made up to the required volume with 70% aqueous methanol. Approximately 2.0 mL of the final solution was filtered through a 0.45 m filter (Millipore Millex-FH, MA U.S.A) and injected into the HPLC.

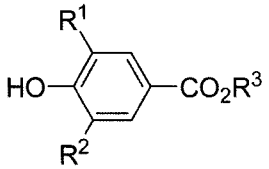
HPLC analysis

The content of the phenolic acids and flavonol aglycones was analyzed using an Agilent HPLC, series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1322 A degasser, a model G1312A binary gradient pump, a model G1329/1330A thermoautosampler, a model G1316 column oven, and an model diode array absorbance detector. An isocratic eluent contained 20% (v/v) acetonitrile in deionized water containing 1% (v/v) formic acid (Rio *et al.*, 2004). A ZORBOX SB-C18 column (4.6 × 250 ㎜) was operated at 30 ℃. The flow rate was 1 mL min^{-1} and the sample injection volume was 10 μL. The UV spectra were recorded from 200 ㎚ to 400 ㎚, and the peak areas were measured at 280 ㎚ for the phenolic acids and at 365 ㎚ for the flavonol aglycones. The chromatographic peaks were identified by comparing their retention time and UV spectra with those of the reference standards. A standard graph for each component was prepared by plotting the concentration versus area. Quantification was carried out from the integrated peak areas of the samples against the corresponding calibration curve. Each sample was analyzed in triplicate.

RESULTS AND DISCUSSION

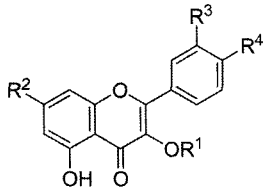
Phenolic acid contents

Currently, a total of four phenolic acids, 4-hydroxybenzoic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), gallic acid (**3**) and



1: $R^1 = R^2 = R^3 = H$
2: $R^1 = OH, R^2 = R^3 = H$
3: $R^1 = R^2 = OH, R^3 = H$
4: $R^1 = R^2 = OH, R^3 = CH_3$

Fig. 1. Phenolic aicds isolated from *Orostachys japonicus* A. Bergers.



5: R^1 = β-D-glucosyl, R^2 = OH, R^3 = H, R^4 = OH
6: R^1 = β-D-galactosyl, R^2 = OH, R^3 = H, R^4 = OH
7: R^1 = α-L-rhamnosyl, R^2 = OH, R^3 = H, R^4 = OH
8: R^1 = α-L-rhamnosyl, R^2 = α-D-glucosyloxy, R^3 = H, R^4 = OH
9: R^1 = β-D-glucosyl, $R^2 = R^3 = R^4$ = OH
10: R^1 = α-L-rhamnosyl, $R^2 = R^3 = R^4$ = OH
12: R^1 = H, R^2 = α-D-glucosyl, $R^3 = R^4$ = OH
13: R^1 = α-D-lyxosyl, $R^2 = R^3 = R^4$ = H

Fig. 2. Flavonol glycosides isolated from *Orostachys japonicus* A. Bergers.

methyl gallate (**4**), were isolated from the *Orostachys japonicus* A. Bergers (**Fig. 1**) (Park *et al.*, 2000).

The content of the four phenolic acids **1-4** in the *Orostachys japonicus* A. Bergers obtained from the night-break and the day-length control experiments was measured in milligrams per kilogram of *Orostachys japonicus* A. Bergers on a dry weight basis, and compared with those in the wild one.

Table 1 gives the content of the phenolic acids **1-4** in the *Orostachys japonicus* A. Bergers according to the cultivation conditions. **Fig. 2** shows a typical chromatogram of the phenolic acids in the wild *Orostachys japonicus* A. Bergers.

The total content of the phenolic acids **1-4** was lowest in the *Orostachys japonicus* A. Berger seedlings (108.3 ㎎/㎏), and highest in the *Orostachys japonicus* A. Berger on the day-length control experiment, 16/8, (483.6 ㎎/㎏). The phenolic acids **1-4** were found in greater amounts in the samples gathered from the night-break experiments than in the wild *Orostachys japonicus* A. Bergers (except for compounds **1** and **3** of the night-break experiment, 8/25).

In the samples cultivated under the day-length control conditions, 13/11 and 10/14, the concentration of compounds **1** and **3** slightly decreased but that of compounds **2** and **4** increased. In the plants cultivated under the day-length control of 16/8, which was the longest day-length, the content of all the phenolic acids **1-4** was remarkably higher. These results show that the production of phenolic acids in *Orostachys japonicus* A. Bergers is light-dependent. These results are in accordance with previous observations showing that the content of phenolic acids in plants increases with increasing light irradiation (Herrma *et al.*, 1976).

Flavonol aglycones contents

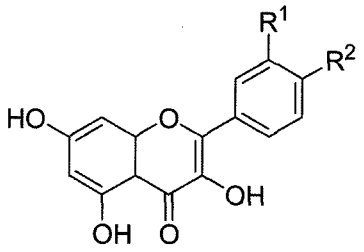
Currently, a total eight flavonol glycosides, four kaempferol glycosides **5-8**, three quercetin glycosides **9-12** and one flavonol glycosides **13** are found in *Orostachys japonicus* A. Bergers (**Fig. 3**) (Park *et al.*, 1989, Park *et al.*, 1991, Park *et al.*, 2000, Sung *et al.*, 2001).

Flavonol glycosides are usually found in plants as *O*-glycosides with sugars bound at the C-3 position (Herrma *et al.*, 1976). The quantitative analysis of flavonol glycosides is performed in the unconjugated form, the flavonol aglycones, process of which includes hydrolysis and extraction followed by HPLC determination, because the reference compounds in the conjugated forms are not readily available (Hkkinen *et al.*, 1998). This offers a practical method for quantifying the flavonoids (Hertog *et al.*, 1993b, Wang *et al.*, 2001).

Table 1. Variation in the concentration (㎎/㎏) of phenolic acids **1-4** in the ***Orostachys japonicus* A. Bergers** according to the cultivation conditions.

Phenolic acid	Wild	Seedling	6/30[a]	7/28[a]	8/25[a]	10/14 [b]	13/11 [b]	16/8 [b]
1	13.3	6.7	16.9	15.9	12.6	11.3	11.2	18.2
2	0.4	6.3	14.3	11.9	2.3	2.3	25.6	20.0
3	214.0	91.9	287.6	387.0	67.0	187.9	207.5	434.5
4	5.9	3.4	9.1	18.6	7.9	8.0	9.8	10.9
Total	233.6	108.3	327.9	433.4	89.8	209.5	254.1	483.6

[a]Night-brake experiment day one, 6/30 = June 30, 7/28 = July 28, 8/25 = August 25; [b]Control of day-length: day/night (hr)



14: R^1 = H, R^2 = OH
15: R^1 = R^2 = OH

Fig. 3. Flavonol aglycones produced by hydrolysis of flavonol glycosides in *Orostachys japonicus* A. Bergers.

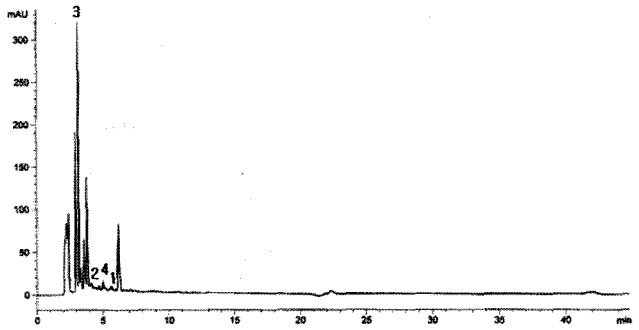


Fig. 4. HPLC chromatogram of the phenolic acids in a wild *Orostachys japonicus* A. Bergers, detection wavelength: 285 nm.

The *Orostachys japonicus* A. Bergers gathered from wild, night-break and day-length controlled experiments were hydrolyzed. The content of the two flavonol aglycones, kaempferol (**14**) and quercetin (**15**), in the night-break and the day-length controlled experiments were measured in terms of milligrams per kilogram dry weight of the *Orostachys japonicus* A. Bergers and compared with those in the wild one. Quercetin is generally regarded as the most important flavonol, followed by kaempferol (**Fig. 4**) (Wang *et al.*, 2001).

Since the completeness of hydrolysis of flavonol glycosides is largely dependent on the type of glycoside and plant tissue (Hertog *et al.*, 1993a, Hertog *et al.*, 1993b), this study investigated the optimum hydrolysis conditions for the *Orostachys japonicus* A. Bergers. **Fig. 5** shows the time for the hydrolysis of the flavonols in the wild *Orostachys japonicus* A. Bergers with the addition of 6 *M* HCl. Both flavonol aglycones **14** and **15** reached their highest yield within 6 h. The influence of three HCl concentrations (2.0, 4.0 and 6.0 *M*) on the hydrolysis of the flavonol glycosides in the *Orostachys japonicus* A. Bergers was investigated. A much longer hydrolysis time was needed to achieve the same result at the lower HCl concentration than at 6 *M* HCl. Based on these two experiments, the

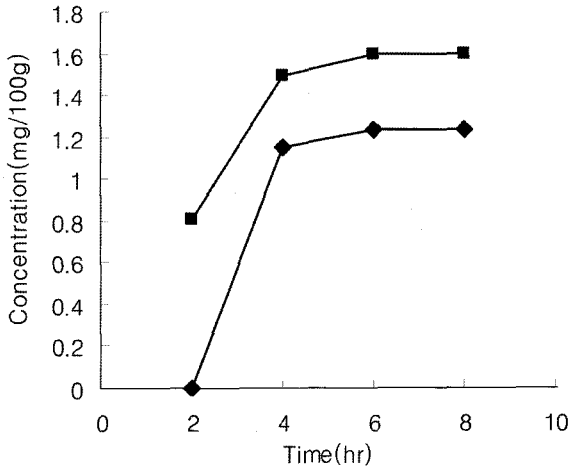


Fig. 5. Hydrolysis course of the flavonols in *Orostachys japonicus* A. Bergers, the values represent the means of triplicate analyses with their standard deviations < 0.1.

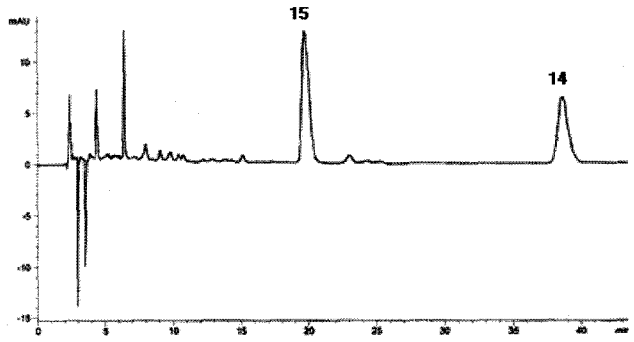


Fig. 6 HPLC of the flavonol aglycones in the hydrolyzed extracts of the wild *Orostachys japonicus* A. Bergers, detection wavelength: 365 nm.

Orostachys japonicus A. Bergers were hydrolyzed in 6 *M* HCl for 6 h. Other acids were not considered because it is well-known that HCl is the most effective for hydrolyzing flavonols (Hertog *et al.*, 1992).

Table 2 shows the quantitative data on the distribution of the flavonol aglycones obtained by hydrolysis of flavonol glycosides in the *Orostachys japonicus* A. Bergers. **Fig. 6** shows a typical chromatogram of the aglycones in the wild *Orostachys japonicus* A. Bergers. The total content of the aglycones **14** and **15** in all the cultivars examined ranged from 18.4 to 35.8 mg/kg. The total distribution of the aglycones **14** and **15** was lowest in the *Orostachys japonicus* A. Bergers from the day-length control experiment 13/11 and highest in the *Orostachys japonicus* A. Bergers from the night-break experiment 7/28. The flavonol aglycones **14** and **15** were found in lower amounts in the *Orostachys japonicus* A. Bergers in the night-break experiment 6/30 and in the day-length control experiment 13/11 than in those of the wild one.

Table 2. Content variation (㎎/㎏) of flavonol aglycones **14** and **15** according to the cultivation conditions.

Flavonol aglycones	Wild	Seedling	6/30[a]	7/28[a]	8/25[a]	10/14 [b]	13/11 [b]	16/8 [b]
14	8.4	10.7	8.3	14.5	11.4	13.4	7.7	14.1
15	12.4	15.0	10.9	21.3	16.8	19.2	10.7	18.6
Total	20.8	25.7	19.2	35.8	28.2	32.6	18.4	32.7

[a]Night-brake experiment day one, 6/30 = June 30, 7/28 = July 28, 8/25 = August 25; [b]Control of day-length: day/night (hr)

The flavonol aglycones **14** and **15** were found in greater amounts in the *Orostachys japonicus* A. Bergers gathered from the experiments irradiated with additional light than in the wild one (except for night-break experiment 6/30 and the day-length control experiment 13/11). These results are in accordance with previous observations showing that the formation of flavonol glycosides in food plants is light-dependent and their content increases with increasing light irradiation (Herrmann *et al.*, 1976).

In conclusion, the two cultivation experiments such as the night-break and the day-length controlling demonstrated that the concentration of phenolic acids **1-4** and flavonol aglycones **14** and **15** in *Orostachys japonicus* A. Bergers increases with the augment of light irradiation.

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LITERATURE CITED

Cheng JT, Hsu FL, Chen HF (1993) Antihypertensive principles from the leaves of melastome candidum. Planta Medica 59:405-407.

Frankel EN, Kanner J, German JB, Parks E, Kinsella JE (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341:454-457.

Gryglewski RJ, Korbut R, Robak J, Swies J (1987) On the mechanism of antithrombotic action of flavonoids. Biochem. Pharmacol 36:317-322.

Harborne JB, Williams CA (2000) Advances in flavonoid research since 992. Phytochemistry 55:481-504.

Herrmann K (1976) Flavonols and flavones in food plants. A review. Journal of Food Technology 11:433-448.

Hertog MGL, Hollman PCH, Venema DP (1992) Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. Journal of Agriculture and Food Chemistry 40:1591-1598.

Hertog MGL, Feskens EJM (1993a) Dietary antioxidant flavonoids and risk of coronary heart disease: the zutphen elderly study. Lancet 342:1007-1011.

Hertog MGL, Hollman PCH, Putte BVD (1993b) Content of potentially anticarcinogenic flavonids of tea infusions. Wine, and fruit juices. Journal of Agriculture and Food Chemistry 41:1242-1246.

Hertog MGL, Feskens EJM, Hollman PCH (1994) Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. Nutrition and Cancer 22:175-184.

Hertog MGL, Kromhout D, Aravanis C (1995) Dietary flavonoids and cancer risk in the zutphen elderly study. Archives of Internal Medicine 155:381-386.

Hertog MGL, Sweetnam PM, Fehily AM, Elwood PC, Kromhout D (1997) Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the caerphilly study. American Journal of Clinical Nutrition 65:1489-1494.

Hkkinen SH, Krenlampi SO, Heinonen IM, Mykknen HM, Trrnen AR (1998) HPLC Method for Screening of Flavonoids and Phenolic Acids in Berries. Journal of the Science of Food and Agriculture 77:543-551.

Kang JH, Park JS, Kim JW (1995) Effect of long-day and night-break treatments on growth and anthesis of *Orostachys japonicus* A. Berger. Korean Journal of Crop Science 40:600-607.

Kang JH, Ryu YS, Cho BG (1996) Effect of night-break period on growth and anthesis of *Orostachys japonicus*. Korean Journal of Crop Science 41:236-242.

Kang JH, Ryu YS, Kang SY, Shim YD, Kim DI (1997) Effect of night-break timing on growth, bolting and anthesis of *Orostachys japonicus*. Korean Journal of Crop Science 42: 597-603.

Park HJ, Young HS, Park JC, Lee SR (1989) Chemical Study on the herbs of *Orostachys japonicus*. Pusan Bulletin of Pharmaceutical Science 23:1-7.

Park HJ, Young HS, Park KY, Rhee SH (1991) Flavonoids from the Whole Plants of *Orostachys japonicus*. Archives of Pharm. Res. 14:167-171.

Park JG, Park JC, Hur JM, Park SJ, Choi DR, Shin DY, Park KY, Cho HW, Kim MS (2000) Phenolic Compounds from *Orostachys japonicus* having Anti-HIV-1 Protease Activity. Natural Product Science 6:117-121.

Price KR, Casucelli F, Colquhoun IJ, Rhodes MJC (1998) Composition and content of flavonol glycosides in broccoli florets (Brassica olearacea) and their fate during cooking. Journal of the Science of Food and Agriculture 77:468-472.

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology & Medicine 20:933-956.

Rio DD, Stewart AJ, Mullen W, Burns J, Lean MEJ, Brighenti F, Crozier A (2004) HPLC-MSnAnalysis of Phenolic

Compounds and Purine Alkaloids in Green and Black Tea. Journal of Agriculture and Food Chemistry 52:2807-2815.

Sung SH, Jung WJ, Kim YC, A (2001) Novel flavonol lyxoside of Orostachys japonicus herb. Natural Product Letters 16:29-32.

Wang H, Helliwell K (2001) Determination of flavonols in green and bleak tea leaves and green tea infusions by high-performance liquid chromatography. Food Research. International 34:223-227.