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Journal of Chromatography B, 846 (2007) 385–390
JOURNAL OF CHROMATOGRAPHY B
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Short communication

An improved HPLC method for the analysis of citrus limonoids in culture media

Qingguo Tian [a], Edward G. Miller [a,b], G.K. Jayaprakasha [a], Bhimanagouda S. Patil [a,*]

[a] *Vegetable and Fruit Improvement Center, Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843-2119, United States*
[b] *Department of Biomedical Science, Baylor College of Dentistry, The Texas A&M University System Health Science Center, Dallas, TX 75246, United States*

Received 30 January 2006; accepted 8 September 2006
Available online 16 October 2006

Abstract

Recent studies have shown that citrus limonoids have potential health benefits. However, information on the absorption and metabolism of limonoids in human gastrointestinal (GI) tract is limited. In the present study we have investigated the metabolism of limonin glucoside (LG), the predominant limonoid in citrus by four microorganisms (*Enterococcus fecalis*, *Escherichia coli*, *Lactobacillus salivarius*, and *Candida albican*) widely present in the human lower GI tract. LG and metabolites in the culture medium were purified using solid phase extraction and analyzed using HPLC using UV detection at 210 nm. The identity of LG was further confirmed by electrospray ionization mass spectrometry (ESI-MS). Significant metabolic activity of *Escherichia coli* and *Candida albican* on LG was observed. Several unidentified metabolites were also found in the medium. The results of the present study indicated that LG may be metabolized in the intestine by some microbes. Further studies are needed to establish the possible route of LG metabolism in the human system.

Keywords: Lower GI tract; Limonoids; HPLC

1. Introduction

Epidemiological evidence has shown that consumption of citrus is protective against a variety of cancers in humans including cancers of the oral cavity, lung, larynx, stomach, esophagus, pancreas, colon, and rectum [1]. Recent animal and cell culture studies also demonstrated the health-promoting properties of certain bioactive compounds in citrus for the prevention of cancer and reduction of cholesterol [2–7]. Limonoids a group of compounds with unique structures are widely present in the Rutaceae and Meliaceae families (Fig. 1). Approximately 37 limonoid aglycones and 19 limonoind glucosides have been identified from citrus and its hybrids [8]. Limonoid aglycones are mainly present in citrus seeds, while limonoid glucosides are present in seeds and fruits at very high concentrations [9]. During last 15 years extensive studies from several laboratories have concentrated on the anticancer activities of citrus limonoids [10]. Our lab and others have been isolating some of these bioactive compounds from different species of citrus to understand their biological activities [2,11–14]. However, the information on the bioavailability and metabolism of limonoids in humans is limited. Recently, limonin was detected in human plasma after ingestion of LG, indicating that LG was possibly hydrolyzed in the intestine and absorbed in the aglycone (limonin) form [15]. Similar to most of flavonoid glucosides, limonoid glucosides could also be hydrolyzed by certain microorganisms in the intestine and absorbed in aglycone forms [15].

The objective of the present study was to investigate the possibility of metabolism of limonin 17-β-D-glucopyranoside (LG) by commonly found microbes in the human lower gastrointestinal (GI) tract. The possible metabolites and LG concentration in the culture media was analyzed by using an improved HPLC method. Electrospray ionization mass spectrometry (ESI/MS) was used to confirm the identity of limonin glucoside.

* Corresponding author. Tel.: +1 979 862 4521/458 8090; fax: +1 979 862 4522.
E-mail address: b-patil@tamu.edu (B.S. Patil).

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doi:10.1016/j.jchromb.2006.09.012

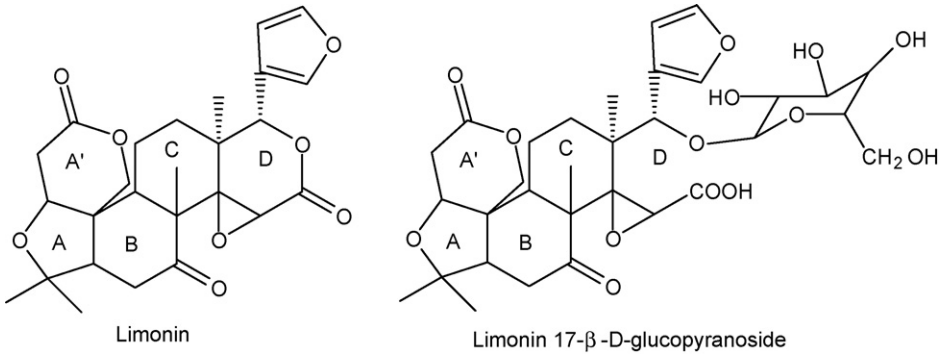

Fig. 1. Structures of limonoids.

2. Materials and methods

2.1. Materials and chemicals

The Sep-Pak C18 cartridges (500 mg/3 ml) were purchased from Waters Associates (Milford, MA, USA). All solvents were obtained from Sigma Chemical Co. (St Louis, MO, USA). Limonin was obtained from Sigma Chemicals Co. (St Louis, MO, USA) and limonin glucoside was obtained from LKT Laboratories Inc. (Minnesota, USA).

2.2. Bacterial cultures

Four microbes, including *Enterococcus fecalis*, *Escherichia coli*, *Lactobacillus salivarius*, and *Candida albican*, were cultured in a medium containing 10 mM of LG. *Enterococcus fecalis*, *Escherichia coli*, and *Candida albican* were grown using brain heart infusion medium while Lactobacilli MRS broth was used for *Lactobacillus salivarius*. The growth of the microorganisms was monitored at every hour. After 24 h incubation, samples were cooled in a refrigerator to 4 °C and centrifuged, Ultra spin cellulose centrifuge filters were used to remove high molecular weight contaminants (MW > 10 kDa). The supernatants were collected for subsequent HPLC analyses. The experiments were carried out in triplicates and averaged.

2.3. Sample preparation

For LG measurement, the medium collected from the experiment was filtered through 0.45 μm Nylon filter (Waters, Milford, MA) and injected into HPLC. A modified solid-phase extraction procedure was used for limonin analysis [16]. The C18 Sep-Pak cartridge was preconditioned by first passing 6 ml of MeOH and water successively for 10 min. An empty syringe was then used to pass air through the cartridge to remove the remaining water. Then, 1 ml of culture medium was passed through the C18 Sep-Pak cartridge for 60 s, and the adsorbed components were eluted with 1.0 ml of dichloromethane, concentrated, and dried under nitrogen. Further, the dried residue was dissolved in 0.1 ml acetonitrile and used for analysis.

2.4. Chromatographic conditions

HPLC analyses were performed on a Thermo Separations HPLC system (Waltham, MA, USA) consisting of spectra system P4000 pump, spectra system AS3000 autosampler and a photo-diode array detector. Chromatographic separations were accomplished on a SPHERISORB ODS-2 column (250 mm × 4.6 mm, 5 μm particle size) (Alltech Associates Inc., Deerfield, IL, USA). The flow rate was set at 1 ml/min and the compounds were detected at 210 nm. LG was eluted using a linear gradient from 10% acetonitrile in 3 mM phosphoric acid to 26% acetonitrile in 3 mM phosphoric acid in 60 min. Limonin was analyzed using a gradient from 10% acetonitrile in 3 mM phosphoric acid to 50% acetonitrile in 3 mM phosphoric acid in 60 min.

2.5. Validation of LC method

(a) Calibration curve and linearity: Calibration curve of LG and limonin was established by injecting 25 μl of LG (equivalent to 10, 20, 30, 40 and 50 μg) and limonin (equivalent to 5, 10, 15, 20, and 25 μg) into the LC system. Concentration of each limonoid versus peak area was used for generating linear regression ($n = 3$).
(b) Range: The calibration range was established through consideration of the practical range necessary according to the use of the LG concentration present in the samples. This range includes concentrations from lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ).
(c) Determination of LLOQ: The LLOQ was defined as the lowest amount of LG and limonin concentration that can be determined with an accuracy and precision of <20%.

2.6. Quantification of LG and limonin

A known volume (25 μl) of the filtered medium and reconstituted solution obtained from SPE were injected to the HPLC. The concentration of LG and limonin (μg/ml) in the culture medium was obtained from their respective calibration curve.

2.7. ESI-MS analysis

The identities of the LG in the cultured medium was confirmed by ESI-MS using Thermo Finnigan LCQ Duo ion trap mass spectrometer operated in both positive and negative electrospray ionization (ESI) modes. Approximately 100 μl of the HPLC eluate separated by a splitter was delivered to the ESI source. The LC conditions were similar to the above HPLC method, except that phosphoric acid was switched to formic acid. Standard limonin and LG in methanol, which was prepared for HPLC analysis, were infused (5 μl/min) for tuning the instrument. The spray voltage was kept at 4.5 kV(±). The capillary temperature was kept at 200 °C. The interface pressure, measured with the convectron gauge at the skimmer cone during electrospray experiments, was normally 0.9 Torr. The base pressure in the ion trap with the added helium was typically 0.9×10^{-5} Torr, as measured with the ionization gauge. The other parameters, including octapole voltages, sheath gas flow rate, and capillary voltage were optimized for maximum intensity of $[M+Na]^+$ and $[M-H]^-$ in positive and negative mode, respectively. The compounds were confirmed based on the molecular weights of the deprotonated molecule species in negative mode and sodium adduct species in positive mode, respectively.

3. Results

3.1. Chromatography

Two HPLC methods were developed for glucoside and aglycone analysis due to the difficulty in separating LG, limonin and other metabolites using single gradient profile. Typical chromatographic profiles for the analysis of standard limonoids and the culture medium of *Candida albican* are shown in Figs. 2 and 3. Baseline separation of LG and limonin was achieved using gradient profiles on a Spherisorb ODS-2 column.

3.2. ESI-MS analysis of the medium

The identity of LG detected in HPLC was further confirmed by ESI-MS (Fig. 4). In the positive and negative ion mode, sample showed $[M+Na]^+$ ion at *m/z* 673.2 and $[M-H]^-$ ion at *m/z* 649.4, which confirms the presence of LG. However, no limonin was detected in the medium of any samples cultured with the four microbes.

3.3. LG concentration changes in the culture medium

There is no obvious LG concentration (0.99 ± 0.02 μg/ml) change before and after the experiment in the medium containing *Lactobacillus salivarius*. However, the LG quantity was significantly ($p < 0.05$) reduced in the culture of the three media containing *Enterococcus fecalis*, *Escherichia coli*, and *Candida albican* (Fig. 5). We could not observe considerable change in LG concentration in the controls (without microbes). In the medium containing *Escherichia coli* and *Candida albican*, only two-thirds (0.675 ± 0.017) and half of the original (0.547 ± 0.033 μg/ml) LG was detected.

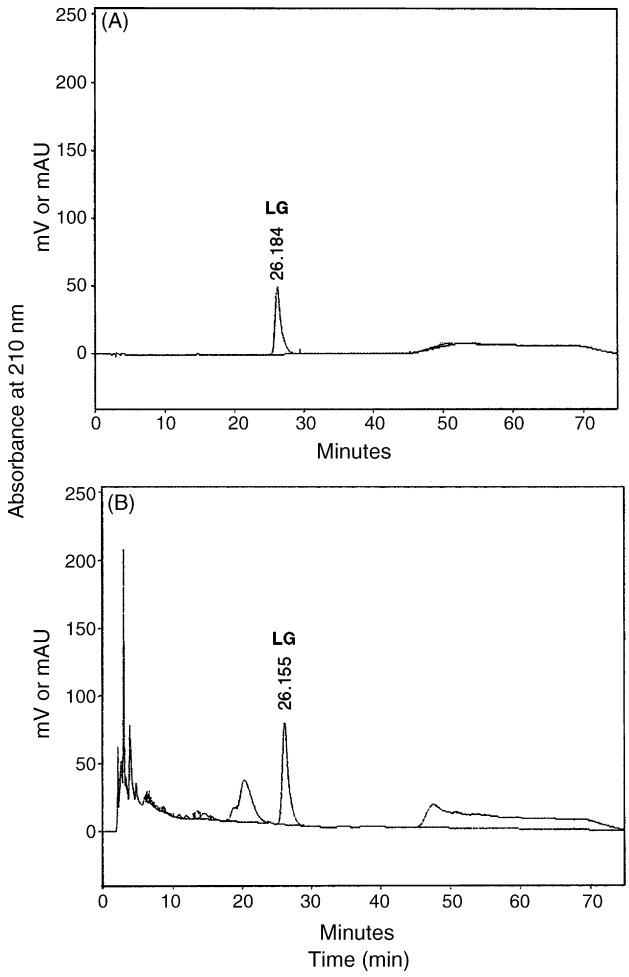

Fig. 2. Typical HPLC chromatograms of (A) standard limonin 17-β-D-glucopyranoside and (B) limonin 17-β-D-glucopyranoside in the culture medium of *Candida albican*.

3.4. Linearity and detection limits of the HPLC analysis

The standard curve was linear up to at least 4.0 mg/ml for LG and 1.6 mg/ml for limonin, respectively. The minimum detectable concentration of the developed method for LG and limonin were 9.5 μg/ml and 4.8 μg/ml, respectively. The detection limit was defined as the lowest concentration of the analyte in the standard, which could be detected from zero with 95% confidence ($n = 10$). Regression analysis of the experimental data points showed a linear relationship with correlation coefficients (r^2) of LG and limonin of 0.975 and 0.984, respectively. The linear regression equations for the curves for LG and limonin (y) are $478.55x - 825.25$ and $125.28x - 228.8$, respectively (Figs. 6 and 7).

4. Discussion

There are number of HPLC methods reported for the quantification of citrus limonoids by HPLC [17,18]. Nuclear magnetic

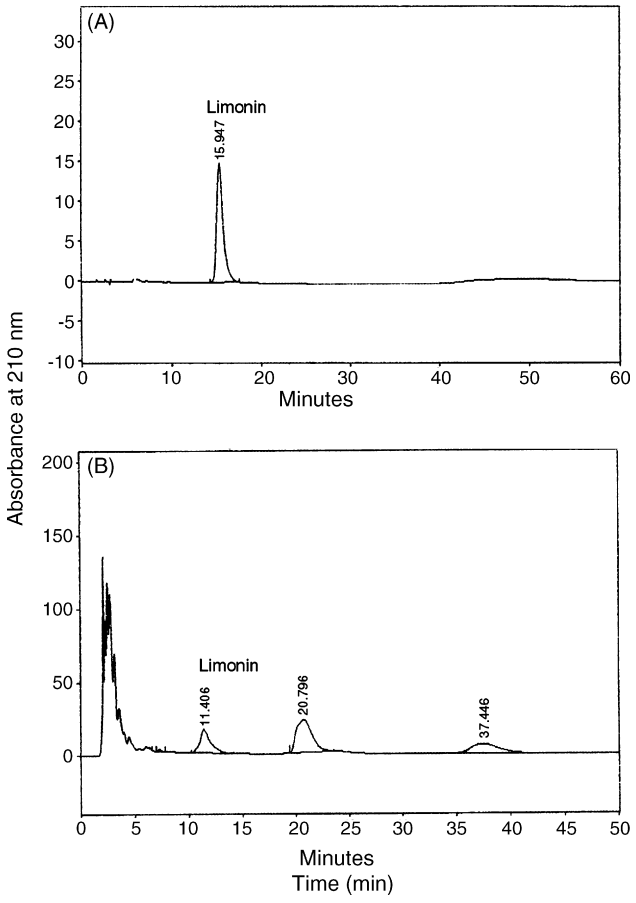

Fig. 3. Representative HPLC chromatograms of (A) limonin and (B) culture media of *Candida albican*.

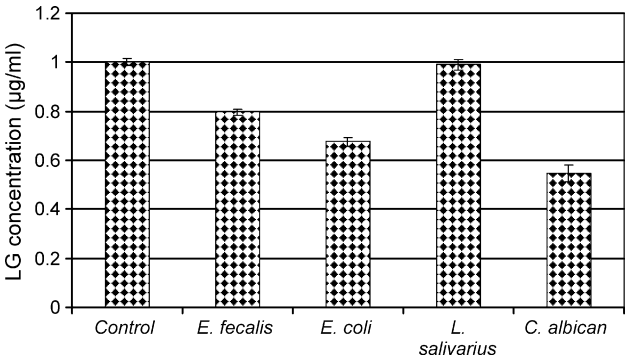

Fig. 5. Concentration of limonin 17-β-D-glucopyranoside in the culture media of four microbes determined by HPLC.

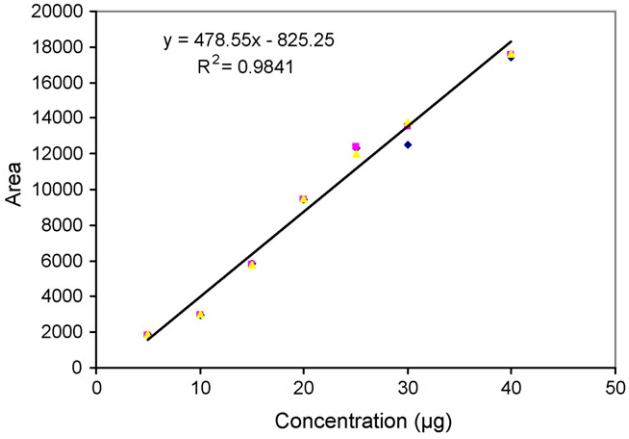

Fig. 6. Calibration graph of limonin.

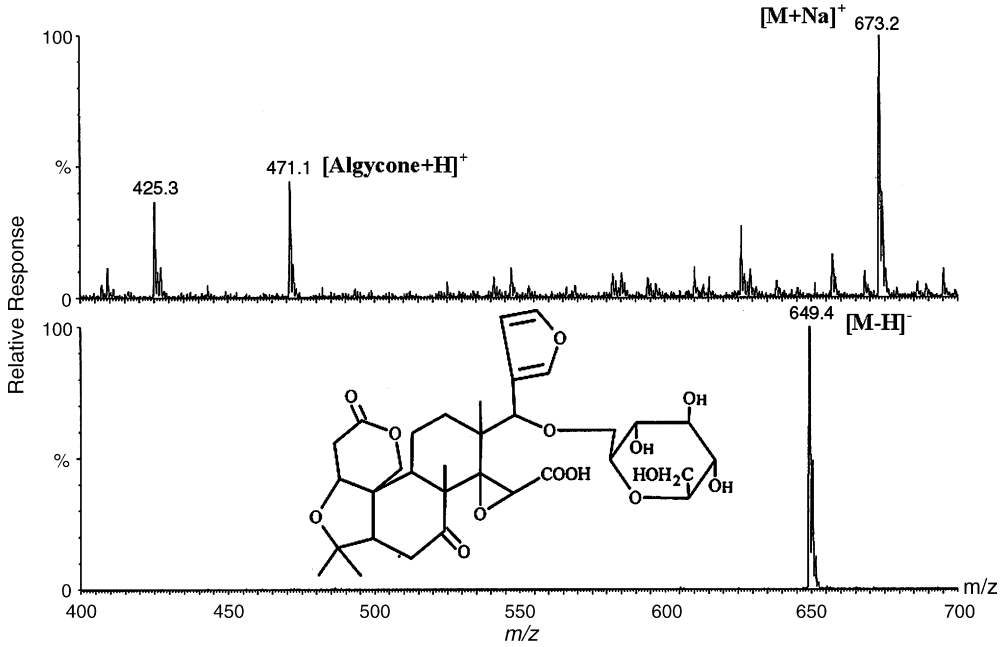

Fig. 4. Positive and negative ESI-MS spectra of limonin 17-β-D-glucopyranoside.

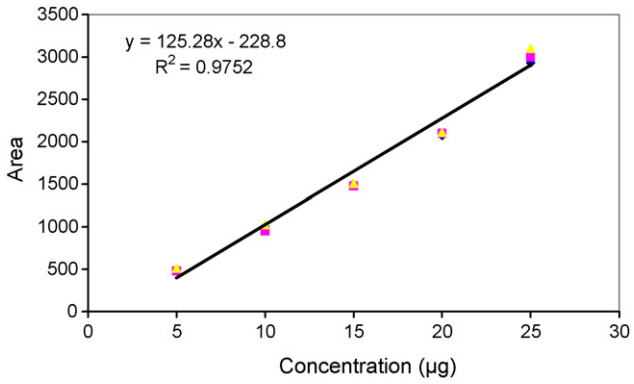

Fig. 7. Calibration graph of limonin glucoside.

resonance (NMR) was often employed for identification of citrus limonoids and it requires extensive and laborious purification. Therefore, additional techniques are needed to confirm the identities of these compounds. Mass spectrometry and tandem mass spectrometry are powerful techniques for identification and structural elucidation of organic compounds by providing not only molecular weight, but also important fragmentation information which may assist in identifying unknown compounds [19]. Tian and Ding [20] reported the application of liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) for screening of limonoid glucosides in citrus and electron ionization mass spectrometry of limonoid aglycones. ESI-MS and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) were also recently used for quantification of limonoid glucosides and aglycones in citrus fruits, respectively [21]. More recently, collisionally activated dissociation (CAD) tandem mass spectrometry (MS/MS) was developed for characterization and structural elucidation of several limonoid aglycones and glucosides [22]. These mass spectrometric techniques allow positive identification of limonoids in complex matrices. Zukas et al. [16] reported the isolation and identification of two metabolites from young Chandler pummelo seedlings. It includes the purification of extracts by column chromatography and preparative HPLC to obtain limonoate A-ring lactone and nomilinoate A-ring lactone. The structures of the isolated limonoinds have been identified using NMR and LC–MS data. However, in the present study, an improved HPLC method and ESI-MS was used to identify limonin and LG in culture samples.

Generally HPLC methods for the analysis of limonoids in citrus fruit samples employs direct application of the samples on the column. This practice gradually reduces the efficiency of the column and shortens its life. However, in the present method the interfering substance such as media was effectively removed by passing the aqueous extract through an octadecyl silane (ODS) cartridge. Subsequently, the cartridge was eluted with suitable solvent and injected to HPLC for the analysis of medium polar compounds. Since, LG is more polar compound than limonin, it was not able to adsorb fully on the cartridge. Hence, we have injected LG containing media to HPLC directly after suitable filtration for the quantification.

Recent studies have showed that limonin was detected in human plasma after ingestion of LG, indicating that LG was possibly hydrolyzed in the intestine and absorbed in the aglycone form [15]. It is possible that the microorganisms in the lower GI tract may hydrolyze LG and release limonin. Our preliminary results indicated that LG in the concentration range of 0.1–10 mM produced no apparent toxic effects on the growth of the four microorganisms. As anticipated, we found that two of the four microorganisms appeared to metabolize LG at a significant level. However, the fact that limonin was not detected in the medium of all samples argues against the assumption that LG was hydrolyzed into limonin and glucose. One possible explanation for this could be that the microbes may have metabolized limonin into other metabolites. A recent study indicated that LG can be metabolized into limonin A-ring lactone (LARL), the precursor of limonin, by cancerous (PC-3) human prostate epithelial cells [15]. However, both LARL and limonin were not detected in the current study, indicating a different metabolic pathway for the breakdown of LG by microbes and cancer cells. Earlier studies on the metabolism of limonoids by bacteria isolated from soil [23–25] may support the explanation of the current study.

Overall this study indicated that LG might be metabolized by microorganisms found in the intestine. These results increase the likelihood that metabolites formed through the breakdown of limonoid glucosides could enter the body in the lower GI tract. To the best of our knowledge, this is the first study to test the metabolism of citrus limonoid glucosides by microorganisms found in the human lower GI tract. Further studies are needed to characterize these metabolites.

Acknowledgements

This project is based upon work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture under Agreement No. 2005-34402-16401, “Designing Foods for Health” through the Vegetable & Fruit Improvement Center and USDA-IFAFS #2001-52102-02294.

References

[1] G. Block, Am. J. Clin. Nutr. 53 (1991) 270S.
[2] S.M. Poulose, E.D. Harris, B.S. Patil, J. Nutr. 135 (2005) 870.
[3] Y. Ozaki, M. Miyake, N. Inaba, S. Ayano, Y. Ifuku, S. Hasegawa, ACS Symposium Series 758 (2000) 107.
[4] J. Vanamala, T. Leonardi, B.S. Patil, S.S. Taddeo, M.E. Murphy, L.M. Pike, R.S. Chapkin, J.R. Lupton, N.D. Turner, Carcinogenesis 27 (2006) 1257.
[5] Y. Liu, H. Ahmad, Y. Luo, D.T. Gardiner, W. McKeehan, R.R. Gunasekara, B.S. Patil, J. Sci. Food Agric. 82 (2002) 469.
[6] E.G. Miller, M.T. Record, W.H. Binnie, S. Hasegawa, Phytochem. Phytopharm. (2000) 95.
[7] Q. Tian, E.G. Miller, H. Ahmad, L. Tang, B.S. Patil, Nutr. Cancer 40 (2001) 180.
[8] S. Hasegawa, M.A. Berhow, G.D. Manners, ACS Symposium Series 758 (2000) 1.
[9] C.H. Fong, S. Hasegawa, Z. Herman, P. Ou, J. Food Sci. 54 (1989) 1505.
[10] S. Ejaz, A. Ejaz, K. Matsuda, C.W. Lim, J. Sci. Food Agric. 86 (2005) 339.

[11] Y. Liu, H. Ahmad, Y. Luo, D.T. Gardiner, W. McKeehan, B.S. Patil, J. Agric. Food Chem. 49 (49) (2001) 3051.
[12] K. Satoru, T. Yasuhiko, O. Kazunori, S. Minoru, Y. Masamichi, Y. Yuko, Anticancer activity and flavonoid content of various citrus juices, in: Nutraceutical Beverages, ACS Symposium Series: American Chemical Society, Washington, DC, 871 (2004) 76–89.
[13] J. Yu, L. Wang, R.L. Walzem, E.G. Miller, L.M. Pike, B.S. Patil, J. Agric. Food Chem. 53 (2005) 2009.
[14] G. Raman, M. Cho, J.S. Brodbelt, B.S. Patil, Phytochem. Anal. 16 (2005) 155.
[15] G.D. Manners, R.A. Jacob, T.K. Breksa III, T.K. Schoch, S. Hasegawa, J. Agric. Food Chem. 51 (2003) 4156.
[16] A.A. Zukas, A.P. Breksa, G.D. Manners, Phytochemistry 65 (2004) 2705.
[17] Y. Ozaki, C.H. Fong, Z. Herman, H. Maeda, M. Miyake, Y. Ifuku, Agric. Biol. Chem. 55 (1991) 137.
[18] H. Ohat, C.H. Fong, M. Berhow, S. Hasegawa, J. Chromatogr. 639 (1993) 295.
[19] K.L. Busch, G.L. Glish, S.A. Luckey, Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry, VCH, New York, 1988, 1–4.
[20] Q. Tian, X. Ding, J. Chromatogr. A 874 (2000) 13.
[21] G.D. Manners, A.P. Breksa, T.K. Schoch, M.B. Hidalgo, J. Agric. Food Chem. 51 (2003) 3709.
[22] Q. Tian, S.J. Schwartz, Anal. Chem. 75 (2003) 5451.
[23] S. Hasegawa, R.D. Bennett, V.P. Maier, J. Agric. Food Chem. 20 (1972) 435.
[24] S. Hasegawa, A.M. Dillberger, G.Y. Choi, J. Agric. Food Chem. 32 (1984) 457.
[25] S. Hasegawa, V.P. Maier, S.N. Border, R.D. Bennett, J. Agric. Food Chem. 22 (1974) 1093.